UNCLASSIFIED

AD NUMBER

ADB274382

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Mar 2001. Other requests shall be referred to U. S. Army Medical Research and Materiel Command, 504 Scott St., Ft. Detrick, MD 21702-5012.

AUTHORITY

USAMRMC ltr, 17 Jun 2002

THIS PAGE IS UNCLASSIFIED

AD_____

Award Number: DAMD17-98-1-8591

TITLE: Potential Prognostic Markers for Human Prostate Cancer

PRINCIPAL INVESTIGATOR: Bruce R. Zetter, Ph.D.

CONTRACTING ORGANIZATION: Children's Hospital
Boston, Massachusetts 02115

REPORT DATE: March 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Mar 01). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020118 181

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-98-1-8591
Organization: Children's Hospital

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

Earl Grant Jr. LTC, MS
20 Dec. 01

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2001	Final (1 Sep 98 - 28 Feb 01)

4. TITLE AND SUBTITLE
Potential Prognostic Markers for Human Prostate Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8591

6. AUTHOR(S)
Bruce R. Zetter, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Children's Hospital
Boston, Massachusetts 02115

E-Mail: Bruce.Zetter@tch.harvard.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Mar 01). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

During the term of this project, we have made significant progress in developing a new prognostic assay for human prostate cancer. Thymosin β15 (TB15) was originally detected in a differential display screen designed to find genes that were upregulated in the later stages of tumor progression. Our studies now show that TB15 expression is extensively upregulated in prostate tumors that are invasive and/or metastatic. By determining the extent of TB15 staining in tumor sections taken at the time of original diagnosis, one can obtain an indication of the likelihood that the tumor will progress to the metastatic state. We have also been able to develop an ELISA for TB15 and have shown that we can detect this protein in the urine of patients with prostate cancer; particularly in patients with recurrent prostate cancer. We are undertaking a prospective study to determine whether urinary TB15 levels at the time of diagnosis can also be predictive of future outcome. Finally, we have begun to develop a collagen-like protein that is upregulated in metastatic prostate cancer as a second prognostic marker. We believe that a panel of such agents will eventually provide an accurate prediction for the likelihood of failure, recurrence or metastasis in newly diagnosed patients.

14. SUBJECT TERMS
Prostate, Prognosis, Markers, Thymosin, Outcomes, PSA failure

15. NUMBER OF PAGES
43

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

Although the development of the PSA test has improved our ability to diagnose prostate cancer, this has not been accompanied by improvements in prostate cancer prognosis. The goal of this project is identify molecules that may be useful as predictive markers for human prostate cancer and to develop these molecules for use in clinically relevant tests that can predict outcome and disease course in prostate cancer patients. Our approach has been to identify molecules that are upregulated late in the course of prostate cancer progression and to then test whether the expression of these markers correlates with disease outcome in patients. Our initial work has been focused on thymosin β15, a molecule found in mid-high grade prostate cancers. We have been following two approaches to develop TB15 testing as a clinically useful approach. The first involves using immunohistochemistry to assay tissue sections from human prostates for the presence of TB15 and to then correlate the expression patterns with PSA failure, tumor recurrence, metastasis or mortality. The second approach has been to attempt to develop an ELISA test to detect TB15 in the urine or serum of prostate cancer patients in order to have a non-invasive assay. We have accomplished both of these goals and our early results do suggest that TB15 may have promise as a marker that can predict later tumor aggressiveness and metastatic potential. We believe that such a test could eventually be used to distinguish those patients at low risk of recurrence from those who are at higher risk. This information could be extremely useful in helping patients and their physicians to choose between the wide range of treatment options currently available.

Body:

In previous studies, by comparing gene expression among the Dunning R-3327 rat prostatic adenocarcinoma variants (1), we cloned a novel β-thymosin gene, thymosin β15, which was expressed in highly metastatic variants, but not in poorly metastatic variant (2). The thymosin β family comprises very small, highly conserved and acidic proteins existing in many different animal species (3). The most abundant member of the family, thymosin β4, which was originally isolated from calf thymus and postulated to play a role in thymic immune development, is present in all mammalian species, and along with the related family member thymosin β10, is widely distributed in a variety of cell types. The main function of thymosins β4 and β10 is to bind monomeric actin and to retard actin polymerization (4)(5). Thymosin β15 also binds monomeric actin and appears to regulate cell motility as transfection of antisense thymosin β15 into rat prostatic carcinoma cells can significantly reduce stimulated cell migration (2).

Note: The figures for this progress report are contained in two presentations that are included in the Appendix and labeled Presentation 1 and Presentation 2.

When we investigated the pattern of expression of TB15 in human prostate cancer we found that low-grade prostate cancers (Gleason 3-5) generally did not express TB15 whereas high-grade tumors (Gleason 8-10) most often had high TB15 levels. What was most interesting, however, was that there was no discernable pattern of TB15 expression in mid-grade tumors. Some were positive for TB15 and some were negative (Presentation 1, Fig. 4). This raised the prospect that TB15 might represent a predictive marker in human prostate cancer, i.e., that tumors that expressed TB15 might be more

aggressive than tumors in which TB15 was not expressed. In Task 1 of this proposal, we further evaluated thymosin β15's use as a potential biomarker that can function as an indicator of metastatic progression and disease outcome in human prostate carcinoma patients by a small-scale retrospective study.

Task 1. Continued Development of Thymosin β15 as a Prognostic Marker in Human Prostate Cancer.

Goals:

a. Continue to accumulate follow-up data on the original cohort of patients analyzed for TB15 expression in our initial studies.

b. Collect and stain tissue specimens for a large (>200 patient) retrospective study correlating TB15 expression with tumor metastasis and patient outcome (Months 6-24).

Results:

A polyclonal antibody was raised against a peptide representing the 11 C-terminal amino acids of thymosin β15. Synthesized peptide was coupled with a carrier, keyhole limpet hemocyanin (KLH), and injected into rabbits. Antiserum was affinity-purified over the C-terminal peptide coupled CNBr-activated sepharose 4B column. To test the specificity of the purified antibody, we performed Western analysis of the GST/thymosin β fusion proteins with the affinity-purified anti C-terminal antibody. The purified antibody reacted strongly with the GST-thymosin β15 fusion protein, but did not cross react with GST-thymosin β4, nor with GST alone. Additional antibodies were prepared to the C-terminal 11 amino acids of Tβ15 by conjugating the peptide to keyhole limpet hemocyanin and injecting the conjugate into chickens. These antibodies showed both high titer and high specificity and did not recognize any other β thymosin isoforms.

We used the affinity purified polyclonal thymosin β15 antibody for immunohistochemical study of 150 human prostate carcinoma cases. Positive immunostaining was observed in the cytoplasm of carcinoma cells in neoplastic prostates but not in normal prostates and not in the stromal cells. Among the specimens investigated, poorly differentiated adenocarcinomas with Gleason scores 8-10 displayed the most extensive and intense thymosin β15 immunoreaction, followed by moderately differentiated prostate carcinomas with Gleason scores of 6-7 in which some but not all carcinomas were TB15 positive. In some cases, specimens of PIN showed thymosin β15 immunostaining, but usually to a lesser extent than the malignant lesions. In poorly differentiated and invasive prostate carcinoma, single cells invading the tissue stroma displayed intense staining. Well-differentiated carcinomas with Gleason scores generally showed no thymosin β15 staining or very low levels of staining.

Thymosin β15 staining levels for all prostatic carcinoma specimens are summarized in Presentation 1, Fig. 4. Specimens were scored as negative if less than 10% of the tumor tissue in each section showed staining of tumor cells, scored as positive (+) if staining was between 10% and 50%, and strong positive (++) if greater than 50% of the tumor tissue was stained. The results show a general correlation between thymosin β15 staining and the Gleason scores. In most cases, high-grade tumors (Gleason scores 8-10) have a higher percentage of positive staining than low-grade (2-5) tumors. Interestingly, moderately differentiated prostate carcinomas with Gleason scores (6-8) could be divided into three groups according to the levels of thymosin β15 expression. 17 (32%) out of 54 cases showed no expression of thymosin β15, about 50% of cells in 24 (44%) cases expressed thymosin β15, showing partial positivity, while 13 (24%) cases showed high levels of thymosin β15 expression (more than 75% cells were positive).

These results suggest that thymosin β15, a potential marker of aggressive prostatic carcinoma, assorts independently in mid-grade prostatic carcinomas.

To further investigate whether TB15 expression correlated with invasion and/or metastasis in human prostate cancer, we conducted a study in collaboration with Dr. John Petros of Emory University Medical School. Tissue samples taken from radical prostatectomy were analyzed for TB15 staining and the staining level (negative -, positive +, strongly positive ++) was recorded and then compared with the invasive or metastatic state of the tumor at the time of prostatectomy. Of the patients with non-invasive disease, 8 were negative, 2 positive and 1 strongly positive for TB15 expression as determined by immunohistochemistry. Patients with invasive but non-metastatic tumors were more generally positive with only 1 of 7 invasive tumors being negative for TB15, 4 positive and 2 strongly positive (Presentation 1, Fig. 5). Finally, all of the 15 patients with metastatic disease diagnosed at the time of surgery were either positive (7 patients) or strongly positive (8 patients).

The follow-up on 26 of patients is summarized in Presentation 1, Fig. 8. Most striking is the data for the nine patients who have died of metastatic prostate cancer (DOD) in the past 3-5 years. Of these, none were negative, one showed positive staining and 8 displayed strongly positive for thymosin β15 at the time of diagnosis. Of the 15 patients still alive with no evidence of disease (NED) 3-5 years following diagnosis, 8 were negative for thymosin β15, three were positive and four were strongly positive for thymosin β15 staining. Thus, of the patients who have follow-up data, all of those patients who were negative for thymosin β15 staining at the time of diagnosis are still

alive with no evidence of disease. Because the population is still only shortly removed from their initial diagnosis, it will be most interesting to monitor whether those patients with no current recurrence but with extensive thymosin β15 staining are indeed at greater risk to develop recurrent disease than those with low thymosin β15 staining.

One of the most interesting analyses of this data is shown in Presentation 1, Fig. 7, which examines tumor recurrence as a function of PSA failure in 72 patients. The reappearance of PSA after removal of the prostate is a clear signal for extraprostatic growth of the tumor. In this figure, it can be seen that PSA failure occurs in 78% of those patients with intermediate Tβ15 staining and 83% of those patients with strong Tβ15 staining. This relationship is shown as a function of time in Presentation 1, Fig. 6 which shows an inverse Kaplan-Meier curve of the percentage of Tβ15 positive and negative patients who go on to PSA failure over time. It can be seen that by 11 years, all of the Tβ15 positive patients have positive PSA levels. In contrast only 25% of the Tβ15 negative patients experience PSA failure. These data again suggest that Tβ15 expression at the time of diagnosis correlates closely with aggressive disease as categorized by eventual PSA failure.

We have distributed our antibodies to thymosin β15 to other laboratories and one such group has published the first data on outcomes in prostate cancer patients following analysis of Tβ15 levels. Chakravarty and colleagues (7) studied 32 patients with clinically localized, moderately differentiated (Gleason 6/10) prostate cancer treated by external beam radiotherapy. All patients had clincal stage M0 disease at initial presentation, which was documented by bone scan. Their corresponding biopsy

speciment were stained immunohistochemically for Tβ15 which was then correlated with the clinical outcome in a blinded test. The median follow-up was 6 years for all of the patients (range = 1-19 years). The outcomes of the 2 patients were grouped into three categories: patients with no evidence of disease (n=11), patients with PSA failure without documented bone failure (n=11) and patients with PSA failure and documented bone failure (n=10). Tβ15 staining intensity strongly correlated with clinical outcome. Of those patients whose specimens stained 3+, 62% developed bone failure compared with 13% of those patients whose specimens stained 1+ (p=.01). The 5-year freedom from PSA failure was only 25% for those patients with 3+ staining compared with 83% for those with 1+ staining (p=.02). The results of this study were taken to demonstrate that Tβ15 staining intensity can identify high and low-risk patients with moderately differentiated, clinically localized prostate cancer.

Task 2. Development of an assay to detect thymosin β15 in human fluids such as urine or serum.

Goals:

a. Develop additional antibodies for TB15 (Month 1-6)

b. Develop a sensitive ELISA or sandwich ELISA assay for TB15

c. Determine whether TB15 can be detected in human fluids (Month 6-12)

d. If TB15 is detected in human fluids, attempt to correlate TB15 expression in patient serum or urine with tumor metastasis and patient outcome (Months 12-30).

It is clear that the utility of the TB15 test would be increased if a non-invasive assay could be developed that could detect TB15 levels in the serum or urine of patients with prostate cancer. The β thymosins are, however, intracellular actin-binding proteins and

they lack a signal peptide for export making it less likely that they would be secreted into human body fluids. Interestingly, though, significant levels of thymosin ?4 are found in the serum of normal individuals (6) suggesting that members of the ?-thymosin family may be released into the circulation. We have therefore attempted to develop an ELISA for thymosin ?15 and to use it to detect TB15 in the urine of prostate cancer patients.

Results:

A polyclonal antibody was raised against a peptide representing the 11 C-terminal amino acids of thymosin ?15. Synthesized peptide was coupled with a carrier, keyhole limpet hemocyanin (KLH), and injected into both rabbits and chickens. Antiserum was affinity-purified over the C-terminal peptide coupled CNBr-activated sepharose 4B column. To test the specificity of the purified antibody, we performed Western analysis of the GST/thymosin ? fusion proteins with the affinity-purified anti C-terminal antibody. The purified antibody strongly reacted with GST-thymosin ?15 fusion protein, but did not cross react with GST-thymosin ?4, nor with GST alone. Using the chicken anti thymosin ?15 antibodies, we have developed an ELISA to TB15 that can detect TB15 at concentrations of 10-20 ng/ml.

The ELISA protocol is as follows:

TB15 (20 ng/well in 200 ?l) was adsorbed to Costar high-binding 96 well plates for 2 h at 37C. The wells were then washed with buffer containing 3 mg/ml BSA and then replaced with 200 ?l of a solution containing a 1:2000 dilution of chicken anti-TB15 antibody along with samples containing urine from prostate cancer patients or from normal controls that had been pre-incubated overnight at 4C. The plates were incubated for 1 h at 37C, then washed 3X, incubated for an additional 1 h with rabbit anti-chicken IgG and finally developed with the Vectastain ABC reagent. A standard curve from a typical assay is shown

in Presentation 2, Fig. 1a. Increasing concentration of TB15 added to the antibody solution results in a progressive decrease in signal with concentrations from 20 ng/ml - 1250 ng/ml.

Our studies have now revealed that TB15 can be detected in the urine of some prostate cancer patients. Figure 1b shows some of the raw data for a group of 11 prostate cancer patients. We have now conducted assays on 120 prostate cancer patients. The only criterion for inclusion in the study was that the patient had been diagnosed as having prostate cancer and was under active care by a urologist. Time following diagnosis ranged from days to decades. Positive TB15 values are ascribed to any patients whose urine TB15 level equals or exceeds 40 ng/ml as determined by extrapolation to a standard curve run on the same day.

As shown in Figure 1c, normal controls are generally negative with 31 patients negative and only 2 positive. Patients previously diagnosed with prostate cancer but disease-free at the time of urine collection were variable in TB15 production with 39 patients positive and 27 patients negative. Since many of these patients were only recently diagnosed, some of these individuals may go on to develop recurrent disease. Of 20 patients who had failed treatment as judged by increasing PSA levels at the time of urine collection, 18 had positive levels of TB15 in their urine whereas only 2 were negative. Finally of three patients who were bone scan positive, 2 were TB15 positive and one was TB15 negative.

We are pleased and surprised that we can detect TB15 in the urine of prostate cancer patients and are currently performing similar studies on a larger sample of 300 patients whom we intend to follow for a five year period. Certainly the trend appears to be that patients with tumor failure have an increased level of detectable TB15 in their

urine. We are now trying to duplicate this work using patient plasma as well as urine samples. Presentation 2, Figure 2 shows that the Tβ15 ELISA assay can detect Tβ15 in human plasma. Figure 2a goes on to show that some prostate cancer patients also have positive levels of Tβ15 in their plasma. Most importantly, we wish to follow this current cohort of patients over time to determine whether newly diagnosed patients who have the highest circulating TB15 levels will be more likely to develop recurrent disease at some later time.

Task 3.

Goals:

Develop new antibodies for the collagen-like protein and obtain antibodies for lipocalin-2 and other potential prognostic markers from investigators (Months 6-12).

Correlate expression of these additional prognostic markers in human prostate cancer tissue specimens with tumor metastasis and patient outcome (Months 12-24).

Develop a sensitive ELISA assay for detection of the collagen-like protein in patient serum and urine (Months 18-24).

If possible, correlate production of the collagen-like protein in patient serum and urine with tumor metastasis and patient outcome (Months 24-30).

Results:

An additional member of the thymosin β family is upregulated in prostate cancer.

TB16. The principal members of the beta-thymosin family are thymosins β4, β10 and β15. The molecules differ markedly in their C terminal 6 amino acids but are highly conserved in the rest of the molecule. Thymosins β4 and β10 are more closely homologous to each other than to thymosin β15. Recently, a fourth mammalian thymosin has been described in the EST database as a beta thymosin isoform found in a

human neuroblastoma. This molecule has been named thymosin NB or thymosin β16 and has been shown to be expressed in human neuroblastoma cell lines. When compared When compared with the other β-thymosins, thymosin β16 shows closer homology to thymosin β15 than to either thymosin β4 or β10. We have raised antibodies to full-length thymosin β16 and show that it cross reacts with thymosin β15 but not with thymosin β4 or β10. In contrast, antibodies to the c-terminal 10 amino acids of thymosin β16 recognize only that isoform and none of the others. We have used the c-terminal antibodies for immunohistochemical staining of human prostate cancer specimens and showed that the distribution of thymosin β16 is similar but not identical to thymosin β15. The principal difference is that thymosin β16 is present more frequently and in lower stage and grade tumors than thymosin β15. One of our principal goals for the future will be to determine the differences between these two closely related isoforms with regard to function as well as to their patterns of expression in different human tumors.

We have also made some progress with our studies on a novel collagen-like gene that is upregulated in certain metastatic prostate cancer cell lines. This molecule has substantial collagen-like repeats along with novel intervening sequences. We have made peptide antibodies to this molecule in chickens using methods similar to those described above for thymosin β15. Using these antibodies in immunohistochemistry on human prostate cancer sections, we find that the protein encoded by this new collagen-like gene (CLG) is also upregulated in aggressive prostate cancers.

The CLG protein is detected in predominantly in higher-grade prostate tumors (Gleason 6 and higher) with the most extensive staining in tumors with Gleason scores of 8 or higher). Further work is being conducted to extend this study and to correlate these

findings to patient outcome. We will also attempt to develop an ELISA for CLG that can be used to detect the protein in patient fluids.

Structural characterization of the thymosin β15 gene. We have now performed detailed sequence analysis of the thymosin β15 gene. The gene appears to exist as a single copy in the rat genome and is comprised of 3 exons distributed over 3 kilobases. The transcription start site was defined by primer extension analysis 30 basepairs upstream from the translation start site. Sequence analysis of the 5'-flanking region upstream of the translation start site revealed domains associated with promoter activity including a GC box, and SP1 site and an egr site. Analysis of the transcriptional activity of the 5'-flanking region was carried out using a luciferase reporter in rat prostate cancer cells. Thymosin β15 sequences fused to the reporter in the appropriate transcriptional orientation consistently produced a ~50-fold increase in luciferase activity when compared to a promoterless luciferase control construct. Thymosin β15 sequences fused in the opposite orientation failed to induce any significant level of reporter activity. This work has now been published (8) and is included in the appendix.

Potential role of thymosin β15 in angiogenesis. Although we primarily see thymosin β15 in the prostate carcinoma cells themselves, we also see some staining in the endothelial cells of small capillaries within the tumors themselves. At first we thought that this was non-specific staining but reports from the Kleinman lab at the NIH has made us think that the staining may be real and relevant. That group has shown that another thymosin family member, thymosin β4, is capable of stimulating endothelial cell migration *in vitro* and angiogenesis and wound healing *in vivo* (9,10). We have confirmed this function for thymosin β15 and show it be capable of both stimulation of endothelial cell migration and of stimulating angiogenesis in the mouse cornea. The

angiogenic activity of thymosin β15 appears to be equal or better than that of thymosin β4. Because thymosin β15 not a component of the normal vasculature, we speculate that thymosin β15 is released from tumor cells and is bound and perhaps internalized by vascular endothelial cells. It is also possible that tumor-derived thymosin β15 potentiates tumor angiogenesis in tumors that produce this molecule.

Key Research Accomplishments:

- ✧ Identified novel potential prognostic markers for human prostate cancer.
- ✧ Developed specific antibodies for potential prognostic markers.
- ✧ Showed that thymosin β15 expression correlates with invasiveness and metastasis in human prostate cancer.
- ✧ Showed that thymosin β15 expression correlates with patient outcome in human prostate cancer.
- ✧ Developed a competitive immunoassay for detection of thymosin β15 in patient urine.
- ✧ Developed a competitive immunoassay for detection thymosin β15 in patient plasma.
- ✧ Demonstrated that thymosin β15 was present in the urine of patients with recurrent prostate cancer.
- ✧ Showed that a novel β-thymosin isoform (thymosin β16) is also upregulated in human prostate cancer.
- ✧ Identified a novel collagen-like gene as a potential prognostic marker in human prostate cancer.

Reportable Outcomes:

Banyard J, Zetter BR. The role of cell motility in prostate cancer. *Cancer Metast. Reviews* 1999; 17:449-458.

Bao L, Loda M, Zetter BR. Thymosin β15 as a prognostic marker for human prostate cancer. Abstracts of the First International Workshop on Thymosin Peptides. 1999.

Hutchinson LM, Short SM, Becker CM, Bakthavachalam S, Zetter BR. Dual functions of Tβ15 in tumor progression: intracellular motility and extracellular angiogenic function. Abstacts of the Keystone Conference on Cell Migration and Invasion. May 2001.

Zetter BR, Hutchinson LM, Bao L. Thymosin β peptides as diagnostic tools in cancer. Abstacts of the International Conference on Immunology and Immunotherapy. May 2001.

Patents:

5,821,033 Human thymosin β15 gene, protein and uses thereof. 11/3/1998

6,017,717 Human thymosin β15 gene, protein and uses thereof. 11/25/2000

5,858,681 Method for prognosis of prostate cancer. 1/12/1999

6,150,117 Method for diagnosis of cancer. 11/21/2000

Individuals receiving pay from this grant

Bruce R. Zetter Ph.D.

Lere Bao Ph.D.

Christian Becker M.D.

Conclusions:

· Thymosin β15 is upregulated in malignant human prostate carcinomas but not in benign tissues.

· Poorly differentiated tumor cells show higher levels of expression of TB15.

· Patients with Tβ15 positive tumors at the time of diagnosis will likely experience PSA failure.

· Tβ15 expression correlates positively with metastatic potential and poor prognosis.

· Thymosin β15 (Tβ15) is detectable in the urine and the plasma of prostate cancer patients

· Preliminary data suggests that TB15 concentration in urine samples is correlated to PSA failure and/or clinical metastatic disease.

· Clinical follow-up data strongly indicate that TB15 could be used as a molecular marker to predict the outcome of prostate cancer patients.

Future Directions:

· Expand retrospective immunohistochemistry study to include 500-1,000 patients.

· Monitor >300 patients continuously over 5 years to determine whether urinary levels of thymosin β15 correlate with PSA failure and metastatic disease.

· Investigate the potential of thymosin β16 and the collagen-like gene (CLG) as potential predictive markers of prostate cancer

References:

1. Isaacs, J. T., Isaacs, W. B., Feitz, W. F., and Scheres, J. Establishment and characterization of seven Dunning rat prostatic cancer cell lines and their use in developing methods for predicting metastatic abilities of prostatic cancers, Prostate. *9:* 261-81, 1986.

2. Bao, L., Loda, M., Janmey, P. A., Stewart, R., Anand-Apte, B., and Zetter, B. R. Thymosin beta 15: a novel regulator of tumor cell motility upregulated in metastatic prostate cancer [see comments], Nat Med. *2:* 1322-8, 1996.

3. Sun, H. Q., Kwiatkowska, K., and Yin, H. L. Actin monomer binding proteins, Current Opinion in Cell Biology. *7:* 102-10, 1995.

4. Huff, T., Zerzawy, D., and Hannappel, E. Interactions of beta-thymosins, thymosin beta 4-sulfoxide, and N-terminally truncated thymosin beta 4 with actin studied by equilibrium centrifugation, chemical cross-linking and viscometry, European Journal of Biochemistry. *230:* 650-7, 1995.

5. Safer, D. and Nachmias, V. T. Beta thymosins as actin binding, Bioessays. *16:* 473-9, 1994.

6. Weller, F. E., Mutchnick, M. G., Goldstein, A. L., and Naylor, P. H. Enzyme iimmunoassay of thymosin beta 4 in human serum, J. Biol. Response Med. *7:* 91-96, 1988.

7. Chakravarit A, Zehr EM, Zietman AL, Shipley W.U. et al. Thymosin Beta 15 predicts for distant failure in patients with clinically localized prostate cancer – Results from a pilot study. Urology 55: 635-638.

8. Bao L, Zetter BR. Molecular cloning and structural characterization of the rat thymosin β15 gene. *Gene*. 2000; 260: 37-44.

9. Malinda, K. M., Goldstein, A. L., and Kleinman, H. K. Thymosin beta 4 stimulates directional migration of human umbilical vein endothelial cells, Faseb J. *11:* 474-81, 1997.

10. Malinda, K. M., Sidhu, G. S., Mani, H., Banaudha, K., Maheshwari, R. K., Goldstein, A. L., and Kleinman, H. K. Thymosin beta4 accelerates wound healing, J Invest Dermatol. *113:* 364-8, 1999.

Appendix

Table I. Thymosin β15 (Tβ15) – A new marker for metastatic prostate cancer

Thymosin β15 is a protein that correlates with metastatic progression in human prostate cancer. We have investigated whether thymosin β15 expression at time of diagnosis can predict disease outcome. Currently there is no independent marker that can predict outcome in human prostate cancer. A 5 year longitudinal study has been completed and the preliminary data has been gathered as summarized below.

- Of 79 patients (Gleason 4-9) with 5 year follow-up after surgery, 59 (75%) had PSA failure an indicator of tumor recurrence
- Tβ15 immunohistochemical staining was performed on paraffin sections of tumors collected immediately after surgery. Staining was determined to be positive or negative.

PSA failure (an indicator of disease recurrence)

TB15 (test)	Failure +	Failure -	Totals
TB15+	53	7	60
TB15-	6*	13	19
Totals	59	20	79

Sensitivity: the proportion of subjects with the disease who have a positive test. In this case, 53/59 (90%) men with recurrent disease had a positive TB15 test at the time of diagnosis.

Specificity: the proportion of subjects without the disease who test negative. In this case, 13/20 (65%) men without recurrent disease had a negative test (*see note below).

Predictive value of a positive test: the probability that a person with a positive test actually has disease recurrence. Here, of the 60 men who tested positive, 53 have recurrent disease (88%).

Predictive value of a negative test: the probability of a person with a negative result does not have disease. Here, 13 of the 19 men with a negative result do not have disease (68%). However, of the 6 patients who were PSA failure+ and Tβ15- (false negative findings), 4 had positive margins and therefore are likely to have had <u>local</u> (non-metastatic) recurrence of the tumor. Because Tβ15 is a predictor of metastatic potential, there is no expected correlation between local regrowth and Tβ15 staining.
**When these patients are removed from the study the specificity of the test improves to 13/15 or 87%.*

- There is a dramatic need for a good predictive test for likelihood of non-local recurrence of prostate cancer after surgery or radiation. The initial results from this retrospective test of patients with 5 year follow-up after treatment for prostate cancer indicate that thymosin β15 expression at time of diagnosis correlates with later disease outcome. Patients who have negative thymosin β15 tests at time of diagnosis may be candidates for watchful waiting. Patients who are thymosin β15 positive at time of diagnosis are at higher risk for tumor recurrence and may be candidates for more aggressive therapy.

ROLE OF THYMOSIN β15 AS A PROGNOSTIC MARKER FOR HUMAN PROSTATE CANCER

Lere Bao[1], Massimo Loda[2], Lloyd Hutchinson[1], Christian Becker[1], and Bruce Zetter[1]

[1]Department of Surgery, Children s Hospital, [2]Department of Pathology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA

BACKGROUND:

- Prostate cancer is the most common malignancy in American men.
- It is a slow-growing tumor with relatively low incidence of mortality.
- If it becomes metastatic, however, prostate cancer is often a fatal disease.
- It is important to distinguish those patients whose tumors will progress to the metastatic state from those with little likelihood of causing mobility.
- Thymosin β15 (TB15) is upregulated in metastatic prostate cancer cells and has a potential as a predictive marker.
- We stained human prostate cancer samples with a TB15 antibody and showed the correlate of TB15 expression with patients outcome.

RESULTS:

Northern Hybridization

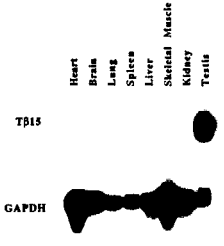


Fig. 1 Expression of TB15 in various normal rat tissues. Northern blot was hybridized with TB15 cDNA probe.

In situ Hybridization

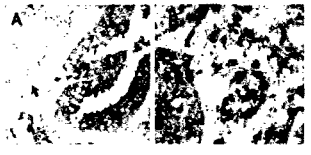


Fig. 2 In situ hybridization with antisense riboprobe for TB15 on human prostate cancer samples. **A.** TB15 mRNA is expressed in select prostatic intraepithelial neoplastic lesions (small arrow), but not others (large arrow). **B.** Invasive single tumor cells display intense TB15 expression.

Immunohistochemistry

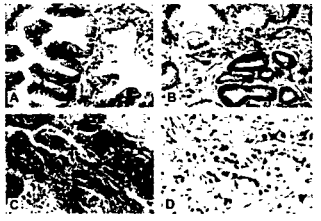


Fig. 3 Immunostaining of human prostate carcinoma tissues with an affinity-purified anti-TB15 C-terminal peptide antibody. **A.** Nonmalignant prostatic epithelia show no TB15 staining (large arrow), occasionally prostatic intraepithelial neoplasia shows weak staining (small arrow). **B.** Moderately differentiated prostate carcinomas show heterogeneous staining in a single prostate (small arrow, positive; large arrow, negative). **C.** Poorly differentiated invasive prostate carcinoma cells show strong TB15 staining. **D.** Single cells invading stroma show intense cytoplasmic staining.

Summary of Immunostaining

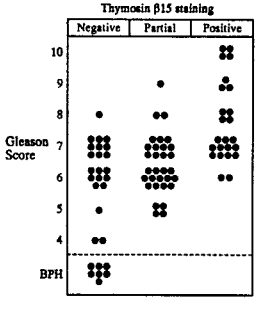


Fig 4 The results are shown for 7 specimens of benign prostatic hyperplasia and 76 cancers with Gleason scores of 4-10. Each symbol represents a specimen from a different individual. Positive, more than 75% of tumor cells showing positivity; partial, 10-75%; negative, less than 10%.

Correlation with Invasion and Metastasis

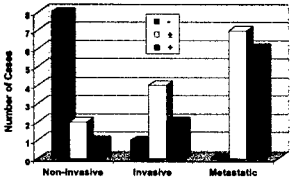


Fig. 5 Correlation of invasive and metastatic potential with initial immunostaining data for TB15 expression in human prostate cancers with 5-year follow-up.

Failure Curve for Margin Negative Patients

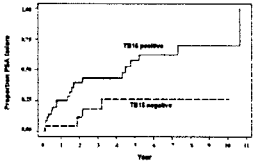


Fig. 6 Correlation of PSA failure data with initial immunostaining data for TB15 expression.

TB15 Staining and Cancer Recurrence

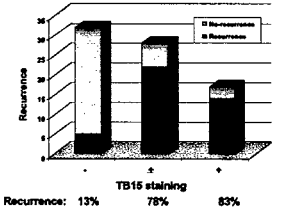


Fig. 7 TB15 positive-prostate cancers show high percentage of recurrence.

SUMMARY:

- TB15 is upregulated in malignant human prostate carcinomas but not in benign tissues.
- Poorly differentiated tumor cells show higher levels of expression of TB15.
- Patients with TB15 positive tumors at the time of diagnosis will likely experience PSA failure.
- TB15 expression correlates positively with metastatic potential and poor prognosis.
- Clinical follow-up data strongly indicate that TB15 could be used as a molecular marker to predict the outcome of prostate cancer patients.

TB15 Staining and Survival Status

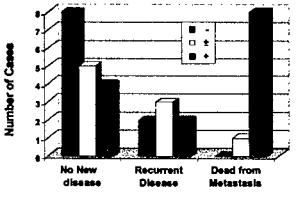


Fig. 8 Correlation of clinical 5-year follow-up survival data with initial immunostaining data for TB15 expression in human prostate carcinoma.

REFERENCES:

1. Bao, L., Loda M., Jammey, P.A., Stewart, R., Anand-Apte, B. and Zetter, B.R. 1996, Thymosin β15: a novel regulator of tumor cell motility upregulated in metastatic prostate cancer. *Nature Med*, **2**, 1322-1328

2. Bao, L., Loda M. and Zetter, B.R. 1998, Thymosin β15 expression in tumor cell lines with varying metastatic potential. *Clin Exp Metastasis*, **16**, 227-233

Presentation 1

243

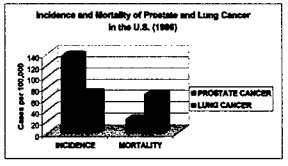
Detection of Thymosin Beta 15 in Urine and Plasma of Prostate Cancer Patients

Christian Becker[1], Eric Chang[1 2], Lloyd Hutchinson[1], Danielle Behonick[1], Lere Bao[1], Jerome Richie[3], Philip Kantoff[0], Bruce Zetter[1]

[1]Children s Hospital, Boston, MA; [2]MD Anderson Cancer Center, Houston; [3]Brigham and Women s Hospital, Boston, MA; [0]Dana Farber Cancer Institute, Boston, MA

Background:

- **Prostate cancer has highest incidence of solid tumors but relatively low mortality.**
- **Thymosin Beta 15 (TB15) is upregulated in human metastatic prostate cancer.**
- **Immunohistochemically it has been shown to be a positive prognostic marker of metastasis.**
- **If detectable in human fluids it might also serve as a diagnostic tool in human prostate cancer.**

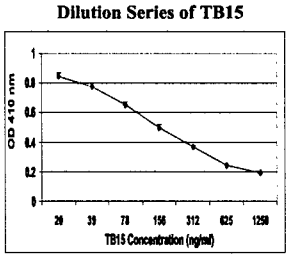


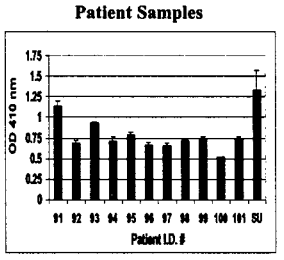
Methods:

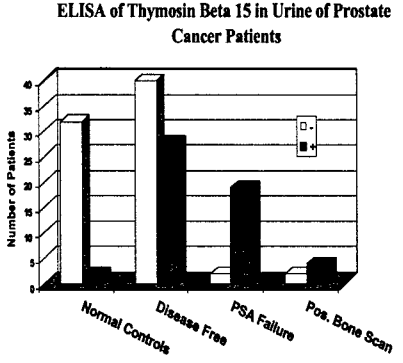
A polyclonal antibody (AB) was raised (chicken) against a peptide representing the 11 C-terminal amino acids of TB15. Urine and plasma samples from prostate cancer patients were randomly collected from two outpatient clinics.
An competition ELISA was developed by coating 96-well plates with GST-TB15 fusion protein. Samples from 344 patients and 20 normal controls were pre-incubated with an AB dilution and then incubated on the plates. Washing steps were followed by incubation with secondary AB (rabbit anti-chicken IgG). The reaction was then developed by an alkaline phosphatase reaction. Dilutions of synthetic TB15 pre-incubated with AB dilution served as a standard.

Results:

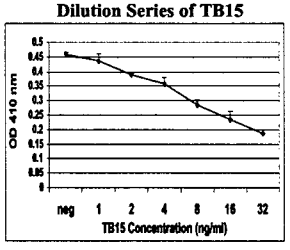
A. Urine:

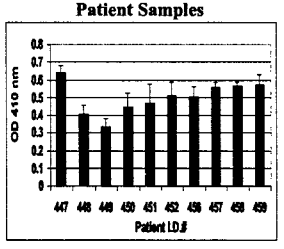






B. Plasma:





Conclusions:

- **Thymosin Beta 15 (TB15) is detectable in the urine and the plasma of prostate cancer patients.**
- **Preliminary data suggests that TB15 concentration in urine samples is correlated to PSA failure and/or clinical metastatic disease.**
- **Thymosin Beta 15 could potentially serve as a diagnostic tool in human prostate cancer.**

Citations:

Bao L, Loda M, Janmey PA, Stewart R, Anand-Apte B, Zetter BR. Thymosin Beta 15: A novel regulator of tumor cell motility upregulated in metatstatic prostate cancer. Nat Med 1996 Dec;2(12):1322-8.

Ries LAG, Kosary CL, Hankey BF, Miller BA, Clegg L, Edwards BK (eds..). SEER Cancer Statistics Review, 1973-1996, National Cancer Institute, Bethesda, MD, 1999.

Presentation 2

Cancer and Metastasis Reviews **17:** 449–458, 1999.
© 1999 *Kluwer Academic Publishers. Printed in the Netherlands.*

The role of cell motility in prostate cancer

Jacqueline Banyard and Bruce R. Zetter
Department of Surgical Research, Children's Hospital, Boston, MA, USA

Key words: prostate cancer, motility, invasion, metastasis

Abstract

Cell motility is a critical determinant of prostate cancer metastasis. The current review discusses the role for cell motility in metastatic dissemination, the evidence that prostate cancer metastasis is dependent on increased cell motility and describes the molecules whose expression has been shown to correlate with the increased motility that accompanies prostate cancer progression. These include receptors for growth factors and cytokines that regulate cell motility as well as intracellular proteins that interact with actin or that regulate signal transduction associated with cell motility. Motility related modulators include both positive regulators of cell movement that are upregulated during tumor progression and suppressors of cell movement that are down-regulated during progression. Because altered expression of such genes may determine the metastatic potential of any particular prostate tumor, we conclude that the appearance or disappearance of motility-related molecules could be used to aid in the diagnosis and prognosis of human prostate cancer.

Tumor cell motility is an important step in the progression of metastasis. Tumor cells migrate from the primary tumor, intravasate into tumor blood vessels, extravasate into the secondary tumor site, and migrate through tissue to establish a secondary metastatic site. Prostate carcinoma preferentially metastasizes to bone and once secondary sites are established, the tumor is aggressive and often associated with a poor prognosis. However, many prostate tumors will remain localized and confined to the prostate indefinitely. Currently there is no means of distinguishing these patients from those in which the prostate tumor will progress to metastasis. This is an important clinical problem as prostate cancer is the second leading cause of cancer death among men in the US. Current tests include prostate serum antigen (PSA), which is a useful determinant of tumor volume but does not reveal metastases or metastatic potential, and histologic grading by Gleason's score [1], which is most useful at the extremes of tumor differentiation. Prediction of the behavior of clinically localized and moderately differentiated prostate tumors remains difficult. As cell migration is a critical step in the metastatic cascade, knowledge of the molecules that enhance or suppress cell motility in prostate cancer cells may lead to new prognostic molecules, as well as new therapeutic targets for the prevention and treatment of metastatic prostate cancer.

A number of studies have demonstrated a strong correlation between tumor cell motility and metastasis. One of the earliest correlations between cellular motility and prostate cancer metastasis was made by Coffey and coworkers in the late 1980's. They utilized a series of prostate carcinoma cell lines of varying metastatic potential that had been established [2] from a spontaneously occurring prostate adenocarcinoma that had been carried by serial subcutaneous transfer in rats since 1963 [3]. This Dunning rat R-3327 prostate cancer cell model has been used extensively to demonstrate the relationship between metastasis and cell motility. Analysis of these cell lines using time lapse microscopy demonstrated that motility, quantified in terms of membrane ruffling, pseudopod extensions and cell translocation, correlates positively with metastatic potential [4,5], see Table 1. Fourier analysis, which determines the temporal and spatial changes in cell contours over time confirmed that cell motility correlates with metastatic potential in these cell lines [6]. The characteristic motility grades of the individual sublines are stably maintained both *in vivo* and *in vitro* [7].

Table 1. Relationship between motility and metastasis in the Dunning R3327 rat prostate adenocarcinoma cell lines [4–7]

Cell subline	Metastatic potential	Motility grade
G	low	low
H	low	low
HIF	low	low
AT1	low	low
AT2	low	low
AT3	high	high
PAT2	high	high
AT6	high	high
MatLu	high	high
MATLyLu	high	high

Growth factor, cytokine and hormonal regulation of prostate carcinoma cell motility

The prostate tumor cell environment is complex, consisting of surrounding tumor cells, extracellular matrix and many other cell types, including endothelial cells, smooth muscle cells, stromal cells and specialized epithelial cells, such as basal and exocrine secretory cells and neuroendocrine cells. Each of these cell types may secrete factors that modulate prostate carcinoma cell behavior, such as proliferation, differentiation and motility. Upregulation of growth factor synthesis or secretion, or altered expression of growth factor receptors on prostate carcinoma cells has the potential to modulate cell motility. For example, production of epidermal growth factor (EGF) and transforming growth factor-α (TGFα) is increased in prostate carcinoma tissue and cell lines [8], and EGF is a potent chemoattractant for several prostate carcinoma cell lines [9]. EGF receptor levels are also increased on prostate carcinoma cells [8], and overexpression of wild type EGF receptor on DU-145 human prostate carcinoma cells increases cell invasion through Amgel (a human basement membrane matrix) [10]. In addition, expression of a truncated, mitogenically active, but motility deficient EGF receptor in DU-145 cells inhibits the metastatic ability of these cells *in vivo* [11], demonstrating that motility, not proliferation, is the key event influencing EGF receptor-induced metastasis. Thus prostate carcinoma cells may increase their motility when they are proximal to EGF-secreting cells. Other factors may also influence EGF or EGF receptor expression. IFN-γ downregulates EGF receptor expression and thereby decreases invasion of DU-145 prostate carcinoma cells through Matrigel (a tumor basement membrane matrix) [12]. Together, these results suggest an important role for EGF receptors in maintaining the motile phenotype of metastatic prostate cancer cells.

Autocrine motility factor (AMF), a prostate tumor cell secreted factor [13], also affects motility of prostate carcinoma cells. Tumor cell migration in response to AMF has been associated with metastatic potential. PC-3M is a highly metastatic subline of the human prostate carcinoma cell line, PC-3, that was established from a human prostate cancer liver metastasis. PC-3M exhibits a higher response to AMF, in terms of phagokinetic track motility analysis and accelerated closure of a experimentally wounded cell monolayer, than the parental PC-3 cells [14]. The AMF receptor, gp78, is differentially expressed on the membranes of PC-3 human prostate carcinoma cells and their highly metastatic subline PC-3M, indicating that receptor expression may be responsible for the differential response of these cells to AMF [14].

Additional growth factors also modulate prostate carcinoma cell motility. These include the transforming growth factor-β (TGFβ), which stimulates migration of MAT-LyLu cells, a highly metastatic subline from the Dunning Rat R33327 prostate carcinoma model, across a porous membrane in an uncoated transwell assay [15]. TGFβ may be produced by metastatic prostate tumor cells, as shown for the highly metastatic AT3 rat prostate adenocarcinoma cell line [16]. However, TGFβ receptors I and II are downregulated in both primary prostate carcinoma and metastatic lesions [17], suggesting that advanced prostate cancer may eventually show TGFβ insensitivity.

Prostate carcinoma frequently metastasizes to bone, a tissue rich in the insulin-like growth factors (IGFs), IGF-I and IGF-II, which are known to stimulate tumor cell movement. Therefore the effect of IGFs on prostate carcinoma cell motility may be important in metastasis. In fact, the chemotactic migration of prostate carcinoma cells is stimulated by either IGF-I or IGF-II [18]. The effect of IGFs on prostate carcinoma cells may also be modulated at the receptor level, as shown for EGF and TGFβ, as the insulin-like growth factor-binding proteins (IGFBPs) are differentially expressed in prostate tumors with high and low Gleason's scores, indicating changes with disease progression [19]. In contrast, two bone-associated growth factors, tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β), decrease human prostate carcinoma cell chemotaxis [18] suggesting that the bone

microenvironment contains both positive and negative modulators of prostate cancer cell motility.

Other cell types present in the prostate tumor environment include neuroendocrine cells. These cells can secrete bombesin, a gastrin-releasing peptide, which increases migration of PC-3 human prostate carcinoma cells [20]. Neuroendocrine cell function includes the secretion of prostatic hormones, which may also influence tumor motility. For example, calcitonin, a hormone involved in calcium regulation increases the chemotactic migration of LNCaP human prostate carcinoma cells [21]. Parathyroid hormone (PTH)-related peptide (PTHrP) is produced by prostatic neuroendocrine cells, and is overexpressed in prostate cancer tissue and cell lines [22]. PTH is a calcium regulating hormone which may be present at sites of prostate cancer bone metastasis that increases chemotactic migration of the human prostate carcinoma cell lines, DU-145 and PC-3 [21]. These experiments suggest that prostate cancer cell motility, and possibly metastasis, could be inhibited by agents that block calcium transients. An additional hormone secreted by the prostate is the androgen, dihydrotestosterone (DHT) which increases translational motility of LNCaP cells [23]. The hormonal status of the prostate, which may change with tumor progression, may thus influence cell motility, suggesting that hormonal therapy for prostate cancer may modulate cell motility and metastasis, in addition to affecting proliferation.

Prostate stromal cells secrete a nerve-growth factor (NGF)-like protein which stimulates the chemotactic and chemokinetic migration of the human prostate tumor cell lines, TSU-Pr1, PC-3 and DU-145 [24]. NGF stimulates DU-145 human prostate carcinoma cell invasion into Matrigel [25]. Hepatocyte growth factor or scatter factor (HGF/SF) is also produced by fibroblasts, and increases cell scattering and Matrigel invasion by DU-145 human prostatic carcinoma cells [26]. These data suggest that the stromal cells surrounding the prostate tumor may influence their migratory behavior.

An additional mechanism of control of prostate tumor cell migration at the primary site may be the paracrine secretion of motility inhibiting factors by tumor cells. For example, the non-motile, non-metastatic G subline in the Dunning R-3327 rat prostate adenocarcinoma system secretes proteins capable of inhibiting motility of the highly motile and metastatic MAT-LyLu subline [27].

It is clear that numerous cell types in the prostate carcinoma environment may secrete factors which can both stimulate and inhibit motility, directly or via modulation of tumor cell receptor expression. Control of these effects may be a mechanism through which tumor cell dissemination may be reduced or prevented, and thus may represent potential targets for therapeutic agents.

Matrix modulation of prostate cancer cell motility

In addition to growth factor and cytokine mediators, the surrounding matrix may affect prostate carcinoma cell motility. Cell adhesion is critical for tumor cells to migrate, and therefore changes in the extracellular matrix may be expected to modulate tumor cell motility. In fact, Matrigel, collagen I and laminin all increase translational motility of LNCaP cells [23], and fibronectin stimulates DU-145 prostate carcinoma cell migration in the Boyden chamber assay [28]. Interestingly, culture of a transformed rat prostate epithelial cell line (NbMC-2) in Matrigel results in altered morphology and increases chemokinetic migration and invasive potential [29]. These data indicate that the extracellular matrix may influence both motility and metastatic progression of prostatic carcinoma cells.

Cell-matrix interactions are mediated via integrin receptors, and changes in integrin receptor expression have been associated with prostate cancer progression. In prostate tumors, increased $\alpha 6$ integrin expression correlates positively with the progression of prostate carcinoma cells to a motile and invasive phenotype. DU-145 cells selected for high (DU-H) and low (DU-L) $\alpha 6$ integrin expression, demonstrate similar adhesion, but the DU-H cells (with 4-fold higher $\alpha 6$ integrin expression), are 3 times more motile on laminin and are more invasive *in vivo* [30]. Integrin receptor $\alpha IIb\beta 3$ may be important in tumor cell dissemination as blocking antibodies inhibit prostate carcinoma cell metastasis [31]. Alternative splicing of integrin receptors is an additional mechanism through which tumor cell interaction with the extracellular matrix can be modulated. Alternative splicing of $\alpha IIb\beta 3$ can result in a truncated receptor which lacks the transmembrane region and cytoplasmic tail of the αIIb light chain. This isoform is detected in the prostate carcinoma cell lines, PC-3 and DU-145, but not in normal prostate epithelial cells [32]. Loss of expression of the $\alpha 4$ integrin subunit

also correlates with the capacity of prostate carcinoma cells to invade through Matrigel [33]. In conclusion, these data suggest that both the extracellular matrix, and receptors mediating cell-matrix contact may play important roles in the control of prostate carcinoma cell movement.

Proteases and cell motility

Secretion of proteases by prostate cancer cells, resulting in degradation of the extracellular matrix, may allow cell migration and invasion into tissues at either the primary site or secondary metastases. The secretion of plasminogen activators (PA), a family of serine proteases, is associated with metastatic potential of prostate carcinoma cell lines. A more aggressive, highly metastatic subline of PC-3 cells, PC-3CALN, secretes 3.5 times more PA activity than the parental PC-3 cells, and invades Matrigel to a greater extent than PC-3 cells. PC-3 metastases also have greater PA activities than the corresponding primary tumor [34], supporting a role for PA in metastasis. Expression of urokinase plasminogen activator (uPA) also correlates with metastatic potential in the Dunning R-3327 rat carcinoma model [35]. Additional extracellular matrix-degrading enzymes, including elastase, a chymotrypsin-like activity, and prostatic hyaluronidase are also elevated in highly metastatic prostate carcinoma cell lines [36,37]. Finally, reduced expression of matrix metalloproteinase-9 (MMP-9 or Gelatinase B) decreases prostate carcinoma cell metastasis while not affecting the growth of the primary tumor [38]. Thus, increased protease activity in prostate carcinoma cells may represent an important mechanism through which cells escape the primary tumor and facilitate increased motility and metastasis.

Clinical compounds affecting prostate carcinoma cell motility

A number of clinical compounds affect prostate carcinoma cell motility. For example, the chemotherapeutic agent Pentosan inhibits motility of the highly metastatic Dunning rat carcinoma subline, MAT-LyLu [39]. Pentosan affects cell-matrix interactions and increases cell ruffling while inhibiting lamellipodal extension and reducing translational migration. Estramustine, an estradiol and nor-nitrogen mustard conjugate anti-microtubule drug, which is used to treat hormone-refractory advanced prostate cancer, inhibits migration of DU-145 prostate carcinoma cells from a cell aggregate and inhibits cell invasion of chick heart fragments [40]. Finally, N-(4-hydroxyphenyl) retinamide (4-HPR or Fenretinide) inhibits migration of PC-3 and TSU-Pr1 human prostate carcinoma cells [41]. Inhibition of cell motility by these clinical compounds may represent an important mechanism through which they prevent metastasis *in vivo*.

Genetic markers of prostate carcinoma encode motility-related proteins

In the past decade, several novel technologies, such as subtractive hybridization, differential display and, most recently, gene arrays, have allowed the identification of several genes whose expression is altered in tumor progression. When these techniques have been applied to the study of prostate cancer, they have not only revealed new metastasis-enhancing and metastasis-suppressing genes, but they have also strengthened the correlation between metastasis and motility. In nearly every case, the molecules that promote prostate cancer metastasis also promote prostate cancer cell motility. Similarly the molecules found to suppress prostate cancer, suppress prostate cancer cell motility, as shown in Table 2. Thus, it appears that the process of prostate cancer progression involves the upregulation of motility promoting genes along with the loss or mutation of motility suppressing genes.

We used differential display to identify a novel gene, upregulated in highly metastatic Dunning Rat carcinoma cells. Thymosin β15 (Tβ15) is a 5.3 kDa member of the beta-thymosin actin-binding protein family. It is expressed in human prostate cancer tissue, with a pattern reflecting increased disease progression as assessed by Gleason grading but is not detected in normal prostate epithelium or in other prostate lesions, such as benign prostate hyperplasia (BPH). We have shown a direct correlation between prostate carcinoma cell motility and Tβ15 expression. Highly motile and highly metastatic AT3.1 cells from the Dunning rat carcinoma model express high levels of Tβ15, and transfection of antisense Tβ15 is sufficient to markedly decrease cell migration [42]. Thus, a molecule that was identified as being upregulated in metastatic prostate cancer cells is necessary for the increased motility of

Table 2. Summary of functional effects of factors on prostate cancer cell motility and metastasis

Molecules		Effect on motility	Association with or effect on metastasis
Growth factors and cytokines	EGF	↑	↑
	AMF	↑	↑
	TGFβ	↑	nd
	IGF-I and IGF-II	↑	nd
	NGF	↑	nd
	HGF/SF	↑	nd
	bombesin	↑	nd
	TNFα	↓	nd
	IL-1β	↓	nd
Hormones	calcitonin	↑	nd
	PTH	↑	nd
	PTHrP	nd	↑
	DHT	↑	nd
Membrane receptors	α6	↑	↑
	α4	↑	nd
	αIIbβ3	↑	↑
	CD44	↓	↓
	E-cadherin	nd	↓
Proteases	plasminogen activators	↑	↑
	elastase	nd	↑
	hyaluronidase	nd	↑
	MMP-9	nd	↑
Actin-binding proteins	Tβ15	↑	↑
	PTEN	↓	↓
	Tmel	nd	↓
Tumor suppressor genes	bcl-2	↑	↑
	KAI-1	↓	↓
	Rb	↓	↓
Intracellular signaling proteins	*ras*	↑	↑
	GAPDH	↑	↑
	PKC-zeta	↓	↓

↑ = increase; ↓ = decrease; nd = not documented.

these cells. The finding that Tβ15 expression correlates with aggressive prostate cancer in humans suggests that it may be useful as a prognostic marker in human prostate cancer. We are currently developing an immunoassay that can detect low levels of Tβ15 in the serum or urine of prostate cancer patients.

Bcl-2 may be an additional positive marker of prostate carcinoma progression. Expression of bcl-2 in prostate carcinoma correlates with increased recurrence of disease [43]. A role for bcl-2 in cancer cell motility was shown in a breast carcinoma cell line, where overexpression of bcl-2 increased both migration and metastasis [44].

In contrast, the tumor suppressor gene PTEN (Phosphatase and Tensin homolog deleted on chromosome 10) is deleted in prostate cancer cell lines [45]. PTEN is a phosphatase with similarity to the cytoskeletal protein, tensin, which binds to actin at focal adhesions and is phosphorylated upon integrin binding. PTEN overexpression inhibits fibroblast cell migration in a wound scrape assay; antisense PTEN expression stimulated cell migration [46]. The migration inhibiting activity is mediated by the interaction of PTEN with focal adhesion kinase (FAK), resulting in reduced focal adhesion formation and cell spreading in a phosphatase-dependent manner. FAK overexpression partially antagonizes the effects of PTEN. Indeed, FAK is highly expressed in metastatic prostate carcinoma [47], and studies have demonstrated a role for FAK in cell migration [48]. Consequently deletion or mutation of PTEN in tumors should result in increased tumor cell motility.

KAI-1 is an additional tumor suppressor gene identified in prostate carcinoma. KAI-1 is a 267 amino acid protein, expressed in normal prostate tissue, but reduced in the prostate carcinoma cell lines PC-3, LNCaP, TSU-Pr1, DU-145 and AT6.1 [49]. Overexpression of KAI-1 in AT6.1 cells suppresses lung metastasis following subcutaneous inoculation, with no effect on the growth rate of the primary tumor. This suggests that the loss of KAI-1 may play a role in metastatic prostate cell dissemination, rather than tumor growth. Like PTEN, KAI-1 is also a suppressor of cell motility (J. Isaacs, personal communication). Thus, two potent cancer metastasis suppressors both function to suppress cell motility. Loss of these genes during prostate cancer progression consequently results in a highly motile, metastatic phenotype.

Loss of the Retinoblastoma (Rb) tumor suppressor gene also correlates with prostate carcinoma progression [50]. Rb-defective cell lines exhibit increased migration across Matrigel in a Boyden chamber assay [51], demonstrating a negative role for Rb in cell motility, as seen for the metastasis suppressor genes, PTEN and KAI-1. Because of its role of a suppressor of both proliferation and migration, Rb may function as both a tumor growth suppressor and a metastasis suppressor.

CD44 may be an additional tumor suppressor gene inactivated in prostate cancer. CD44 is a transmembrane glycoprotein, involved in cell adhesion by acting as the receptor for the extracellular matrix molecule, hyaluronic acid. CD44 can be alternatively spliced and loss of either the standard or variant isoforms is associated with prostate cancer progression [52]. Downregulation of CD44 expression also correlates with metastatic potential in the Dunning rat prostate carcinoma model, and overexpression in the highly metastatic AT3.1 cells reduces lung metastasis without affecting proliferation rate [53]. Similarly, overexpression of CD44 in PC-3 human prostate carcinoma cells, which express very low CD44 levels, inhibits metastasis *in vivo* [54]. Interestingly, inhibition of metastasis by CD44 overexpression was recently shown to be independent of hyaluronan binding [53], suggesting that CD44 may mediate an additional function required in metastasis.

Lastly, decreased expression of E-cadherin, a member of the family of epithelial cell–cell calcium-dependent adhesion molecules, is found in invasive sublines of the Dunning R-3327 rat prostate adenocarcinoma model [55]. Decreased cell–cell contact with surrounding prostate epithelial cells may facilitate carcinoma cell dissemination by allowing cells to disaggregate and separate from the primary tumor.

The relationship between cell motility and metastatic potential has been demonstrated for numerous other marker genes, including nm23 [56], TIMP-2 [57], and MTS-1, a member of the S100 family of Ca^{2+} binding proteins [58]. A number of tumor markers have been identified in prostate carcinoma, including MTS-1 (CDKN2) [59,60], DCC, APC/MCC, WAF1/CIP1 [61], and PTI-1 [62], but their role in prostate carcinoma cell motility has not yet been documented. The roles of genetic tumor markers in prostate cancer have been previously reviewed [63,64]. We expect that the majority of metastasis-related genes will encode proteins that have some influence on tumor cell motility.

Modulation of intracellular function in prostate cancer

Cell migration depends, in part, on the regulation of intracellular calcium fluxes. The concentration of extracellular calcium modulates PC-3 human prostatic carcinoma cell migration in the Boyden chamber assay, whereas culturing cells in low calcium decreased invasion across a basement membrane-coated filter. Exposure to higher calcium levels stimulates invasion in a dose-dependent manner [65]. Several actin-binding proteins that influence cell movement are sensitive to local calcium concentrations. For example, human epithelial tropomyosin (Tme1), a calcium dependent actin-binding protein, is downregulated in human prostate carcinoma cells compared to normal prostate epithelium [66]. Down-regulation of tropomyosin with increased tumorigenicity indicates that tropomyosin function may be required for regulation of the actin cytoskeleton during cell motility.

The control of ionic influx affects cell physiology, and altered expression of cell surface transporter proteins is associated with progression of tumor cells to a motile, metastatic state. Metastatic ability has been correlated with the expression of sodium channel proteins in prostate carcinoma cells. Cell transfection of rat prostatic carcinoma genomic DNA also results in elevated levels of sodium channel protein expression [67]. These authors suggest that either intracellular ionic homeostasis or membrane electrophysiological changes may explain the observed effect on cell invasion.

Intracellular signaling molecules mediate the transduction of growth factor and extracellular matrix

signals, and these signals modulate prostate carcinoma cell motility and metastasis. It is now well established that deregulation of signaling molecule expression can affect cell motility. The expression of the small GTPase p21 *ras* shows a strong positive correlation with histologic grade in prostate cancer tissues [68], and expression of v-*ras* in the poorly motile, poorly metastatic AT2 Dunning rat prostate carcinoma cell line increases motility and metastasis [69]. Increased expression of Ha-*ras* is also associated with increased metastasis in one lineage of the Dunning R-3327 rat prostatic carcinoma model [70].

Expression of the signaling molecule protein kinase C (PKC)-zeta negatively correlates with prostate cancer cell motility. PKC-zeta is downregulated in highly metastatic Dunning rat carcinoma cell lines. Conversely, PKC-zeta overexpression in highly metastatic MAT-LyLu cells decreases invasion through Matrigel and the formation of lung metastases *in vivo* [71], suggesting that PKC-zeta may represent an important intracellular signaling checkpoint in the control of prostate carcinoma metastasis.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression is positively associated with increased motility and metastasis in the Dunning rat prostatic carcinoma model [72]. GAPDH has diverse biological functions and the mechanism by which increased expression affects cell motility has not yet been reported.

The experimental record thus demonstrates that several intracellular molecules that are capable of modulating tumor cell motility also influence metastasis. The loss and acquisition of these motility-related proteins is a critical determinant of metastatic potential.

Conclusion and therapeutic implications

Tumor cell motility is a critical step during the progression of tumor cells to the metastatic phenotype. As we have reviewed, the motility of prostate carcinoma cells can be influenced by numerous factors in the tumor environment, including growth factors, hormones and extracellular matrix. Blocking migration stimuli or delivering motility inhibitors are obvious steps for possible clinical intervention. For example, if means could be found to prevent the loss of motility/metastasis suppressing proteins such as PTEN or KAI-1, or to prevent the gain of motility/metastasis enhancing molecules such as thymosin β15, progression to the metastatic state could be delayed.

Perhaps the most exciting area of current research that may lead to new clinical tools and therapeutic agents is the identification of genes that are differentially expressed with prostate cancer progression. Expression of molecules that either promote or suppress cell motility and metastasis define a specific axis through which tumor progression might be monitored. A large proportion of prostate tumors remain localized to the prostate indefinitely and for such patients 'watchful waiting' may be more appropriate than radical prostatectomy and/or radiation therapy. Assays detecting the presence or absence of genetic markers of disease progression, such as these motility-modulating molecules, would provide a means of characterizing the metastatic potential of the prostate tumor, which could in turn influence treatment decisions. The ability to distinguish indolent tumors from aggressive tumors could have the potential to individualize cancer treatment for each patient.

Acknowledgement

We acknowledge support from the NIH.

References

1. Gleason DF, Mellinger GT: Prediction of prognosis for prostate adenocarcinoma by combined histological grading and clinical staging. J Urol 111: 58–64, 1974
2. Isaacs JT, Isaacs WB, Feitz WFJ, Scheres J: Establishment and characterization of seven Dunning rat prostatic cancer cell lines and their use in developing methods for predicting metastatic abilities of prostatic cancers. Prostate 9: 261–281, 1986
3. Dunning WP: Prostatic cancer in the rat. Natl Cancer Instit Monogr 12: 351–369, 1963
4. Mohler JL, Partin AW, Coffey DS: Prediction of metastatic potential by a new grading system of cell motility: validation in the Dunning R-3327 prostatic adenocarcinoma model. J Urol 138: 168–170, 1987
5. Mohler JL, Partin AW, Isaacs JT, Coffey DS: Metastatic potential predication by a visual grading system of cell motility: prospective validation in the Dunning R-3327 prostatic adenocarcinoma model. Cancer Res 48: 4312–4317, 1988
6. Partin AW, Schoeniger JS, Mohler JL, Coffey DS: Fourier analysis of cell motility: correlation of motility with metastatic potential. Proc Natl Acad Sci USA 86: 1254–1258, 1989
7. Doyle GM, Sharief Y, Mohler JL: Prediction of metastatic potential by cancer cell motility in the Dunning R-3327 prostatic adenocarcinoma *in vivo* model. J Urol 147: 514–518, 1992

8. Ching KZ, Ramsey E, Pettigrew N, D'Cunha R, Jason M, Doss JG: Expression of mRNA for epidermal growth factor, transforming growth factor-alpha and their receptor in human prostate tissue and cell lines. Mol Cell Biochem 126: 151–158, 1993
9. Zolfaghari A, Djakiew D: Inhibition of chemomigration of a human prostatic carcinoma cell (TSU-Pr1) line by inhibition of epidermal growth factor receptor function. Prostate 28: 232–238, 1996
10. Xie H, Turner T, Wang M-H, Singh RK, Siegel GP, Wells A: *In vitro* invasiveness of DU-145 human prostate carcinoma cells is modulated by EGF receptor-mediated signals. Clin Exp Metast 13: 407–419, 1995
11. Turner T, Chen P, Goodly LJ, Wells A: EGF receptor signaling enhances *in vivo* invasiveness of DU-145 human prostate carcinoma cells. Clin Exp Metast 4: 409–418, 1996
12. Hobeika AC, Etienne W, Cruz PE, Subramaniam PS, Johnson HM: IFNgamma induction of p21WAF1 in prostate cancer cells: role in cell cycle, alteration of phenotype and invasive potential. Int J Cancer 77: 138–145, 1998
13. Evans CP, Walsh DS, Kohn EC: An autocrine motility factor secreted by the Dunning R-3327 rat prostatic adenocarcinoma cell subtype AT2.1. Int J Cancer 49: 109–113, 1991
14. Silletti S, Yao JP, Pieta KJ, Raz A: Loss of cell-contact regulation and altered responses to autocrine motility factor correlate with increased malignancy in prostate cancer cells. Int J Cancer 63: 100–105, 1995
15. Morton DM, Barrack ER: Modulation of transforming growth factor beta 1 effects on prostate cancer cell proliferation by growth factors and extracellular matrix. Cancer Res 55: 2596–2602, 1995
16. Matuo Y, Nishi N, Takasuka H, Masuda Y, Nishikawa K, Isaacs JT, Adams PS, McKeehan WL, Sato GH: Production and significance of TGF-beta in AT-3 metastatic cell line established from the Dunning rat prostatic adenocarcinoma. Biochem Biophys Res Commun 166: 840–847, 1990
17. Guo Y, Jacobs SC, Kyprinou N: Down-regulation of protein and mRNA expression for transforming growth factor-beta (TGF-beta 1) type I and type II receptors in human prostate cancer. Int J Cancer 71: 573–579, 1997
18. Ritchie CK, Andrews LR, Thomas KG, Tindall DJ, Fitzpatrick LA: The effects of growth factors associated with osteoblasts on prostate carcinoma proliferation and chemotaxis: implications for the development of metastatic disease. Endocrinology 138: 1145–1150, 1997
19. Figueroa JA, De Raad S, Tadlock L, Speights VO, Rinehart JJ: Differential expression of insulin-like growth factor binding proteins in high versus low Gleason score prostate cancer. J Urol 159: 1379–1383, 1998
20. Aprikian AG, Tremblay L, Han K, Chevalier S: Bombesin stimulates the motility of human prostate-carcinoma cells through tyrosine phosphorylation of focal adhesion kinase and of integrin-associated proteins. Int J Cancer 72: 498–504, 1997
21. Ritchie CK, Thomas KG, Andrews LR, Tindall DJ, Fitzpatrick LA: Effects of the calciotrophic peptides calcitonin and parathyroid hormone on prostate cancer growth and chemotaxis. Prostate 30: 183–187, 1997
22. Wu G, Iwamura M, di Sant'Agnese PA, Deftos LJ, Cockett AT, Gersahgen S: Characterization of the cell-specific expression of parathyroid hormone-related protein in normal and neoplastic prostate tissue. Urology (51; 5A Suppl): 110–120, 1998
23. Murphy BC, Pienta KJ, Coffey DS: Effects of extracellular matrix components and dihydrotestosterone on the structure and function of human prostate cancer cells. Prostate 20: 29–41, 1992
24. Djakiew D, Pflug BR, Delsite R, Onoda M, Lynch JH, Arand G, Thompson EW: Chemotaxis and chemokinesis of human prostate tumor cell lines in response to human prostate stromal cell secretory proteins containing a nerve growth factor-like protein. Cancer Res 53: 1416–1420, 1993
25. Geldof AA, De Kleijn MA, Rao BR, Newling DW: Nerve growth factor stimulates *in vitro* invasive capacity of DU145 human prostatic cancer cells. J Cancer Res Clin Oncol 123: 107–112, 1997
26. Nishimura K, Kitamura M, Takeda S, Nonomura N, Tsujimura A, Matsumiya K, Miki T, Matsumoto K, Okuyama A: Regulation of invasive potential of human prostate cancer cell lines by hepatocyte growth factor. Int J Urol 5: 276–281, 1998
27. Mohler JL, Bakewell WE, Sharief Y, Coleman WB, Chay CH, Silvers SM, Smith GJ: Detection of candidates for cancer cell motility inhibitory protein in the Dunning adenocarcinoma model. Clin Exp Metast 13: 474–480, 1995
28. Hullinger TG, McCauley LK, DeJoode ML, Somerman MJ: Effect of bone proteins on human prostate cancer cell lines *in vitro*. Prostate 36: 12–22, 1998
29. Freeman MR, Bagli DJ, Lamb CC, Guthrie PD, Uchida T, Slavin RE, Chung LW: Culture of a prostatic cell line in basement membrane gels results in an enhancement of malignant properties and constitutive alterations in gene expression. J Cell Physiol 158: 325–336, 1994
30. Rabinovitz I, Nagle RB, Cress AE: Integrin alpha6 expression in human prostate carcinoma cells is associated with a migratory and invasive phenotype *in vitro* and *in vivo*. Clin Exp Metast 13: 481–491, 1995
31. Trikha M, Raso E, Cai Y, Fazakas Z, Paku S, Porter AT, Timar J, Honn KV: Role of alpha II (b)beta3 integrin in prostate cancer metastasis. Prostate 35: 185–192, 1998
32. Trikha M, Cai Y, Grignon D, Honn KV: Identification of a novel truncated alpha IIb integrin. Cancer Res 58: 4771–4775, 1998
33. Haywood-Reid PL, Zipf DR, Springer WR: Quantification of integrin subunits on human prostatic cell lines: comparison of nontumorigenic and tumorigenic lines. Prostate 31: 1–8, 1997
34. Gaylis FD, Keer HN, Wilson MJ, Kwaan HC, Sinha AA, Kozlowski JM: Plasminogen activators in human prostate cancer cell lines and tumors: correlation with the aggressive phenotype. J Urol 142: 193–198, 1989
35. Quax PH, de Bart AC, Schalken JA, Verheijen JH: Plasminogen activator and matrix metalloproteinase production and extracellular matrix degradation by rat prostate cancer cells *in vitro*: correlation with metastatic behavior *in vivo*. Prostate 32: 196–204, 1997

36. Lowe FC, Isaacs JT: Biochemical methods for predicting metastatic ability of prostatic cancer utilizing the Dunning R-3327 rat prostatic adenocarcinoma system as a model. Cancer Res 44: 744–52, 1984
37. Lokeshwar VB, Lokeshwar BL, Pham HT, Block NL: Association of elevated levels of hyaluronidase, a matrix-degrading enzyme, with prostate cancer progression. Cancer Res 56: 651–657, 1996
38. Sehgal G, Hua J, Bernhard EJ, Sehgal I, Thompson TC, Muschel RJ: Requirement for matrix metalloproteinase-9 (gelatinase B) expression in metastasis by murine prostate carcinoma. Am J Pathol 152: 591–596, 1998
39. Pienta KJ, Murphy BC, Isaacs WB, Isaacs JT, Coffey DS: Effect of Pentosan, a novel cancer chemotherapeutic agent, on prostate cancer cell growth and motility. Prostate 20: 233–241, 1992
40. Mareel MM, Storme GA, Dragonetti CH, De Bruyne GK, Hartley-Asp B, Segers JL, Rabaey ML: Antiinvasive activity of estramustine on malignant MO4 mouse cells and on DU-145 human prostate carcinoma cells *in vitro*. Cancer Res 48: 1842–1849, 1988
41. Kim JH, Tanabe T, Chodak GW, Rukstalis DB: *In vitro* anti-invasive effects of N-(4-hydroxyphenyl)-retinamide on human prostatic adenocarcinoma. Anticancer Res 15: 1429–1434, 1995
42. Bao L, Loda M, Janmey PA, Stewart R, Anand-Apte B, Zetter BR: Thymosin β15: a novel regulator of tumor cell motility upregulated in metastatic prostate cancer. Nature Med 2: 1322–1328, 1996
43. Keshgegian AA, Johnston E, Cnaan A: Bcl-2 oncoprotein positivity and high MIB-1 (Ki-67) proliferative rate are independent predictive markers for recurrence in prostate carcinoma. Am J Clin Pathol 110: 443–449, 1988
44. Del Bufalo D, Biroccio A, Leonetti C, Zupi G: Bcl-2 overexpression enhances the metastatic potential of a human breast cancer line. FASEB J 11: 947–953, 1997
45. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R: PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275: 1943–1947, 1997
46. Tamura M, Gu J, Matsumoto K, Aota S, Parsons R, Yamada K: Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor, PTEN. Science 280: 1614–1617, 1998
47. Tremblay L, Hauck W, Aprikian AG, Begin LR, Chapdelaine A, Chevalier S: Focal adhesion kinase (pp125FAK) expression, activation and association with paxillin and p50CSK in human metastatic prostate carcinoma. Int J Cancer 68: 164–171, 1996
48. Zachary I: Focal adhesion kinase. Int J Biochem Cell Biol 29: 929–934, 1997
49. Dong J-T, Lamb PW, Rinker-Schaeffer CW, Vukanovic J, Ichikawa T, Isaacs JT, Barrett JC: KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. Science 268: 884–886, 1995
50. Theodorescu D, Broder SR, Boyd JC, Mills SE, Frierson HF Jr: p53, bcl-2 and retinoblastoma proteins as long term prognostic markers in localized carcinoma of the prostate. J Urol 158: 131–137, 1997
51. Li J, Hu SX, Perng GS, Zhou Y, Xu K, Zhang C, Seigne J, Benedict WF, Xu HJ: Expression of the retinoblastoma (RB) tumor suppressor gene inhibits tumor cell invasion *in vitro*. Oncogene 13: 2379–2386, 1996
52. Kallakury BV, Yang F, Figge J, Smith KE, Kausik SJ, Tacy NJ, Fisher HA, Kaufman R, Figge H, Ross JS: Decreased levels of CD44 protein and mRNA in prostate carcinoma. Correlation with tumor grade and ploidy. Cancer 78: 1461–1469, 1996
53. Gao AC, Lou W, Sleeman JP, Isaacs JT: Metastasis suppression by the standard CD44 isoform does not require the binding of prostate cancer cells to hyaluronate. Cancer Res 58: 2350–2352, 1998
54. Miyake H, Hara I, Okamoto I, Gohji K, Yamanaka K, Arakawa S, Saya H, Kamidono S: Interaction between CD44 and hyaluronic acid regulates human prostate cancer development. J Urol 160: 1562–1564, 1998
55. Bussemakers MJG, van Moorselaar RJA, Giroldi LA, Ichikawa T, Isaacs JT, Takeichi M, Debruyne FMJ, Schalken JA: Decreased expression of E-cadherin in the progression of rat prostatic cancer. Cancer Res 52: 2916–2922, 1992
56. Kantor JD, McCormick B, Steeg PS, Zetter BR: Inhibition of tumor motility after nm23 transfection of human and murine tumor cells. Cancer Res 53: 1971–1973, 1993
57. Ray JM, Stetler-Stevenson WG: TIMP-2 expression modulates human melanoma cell adhesion and motility. Ann N Y Acad Sci. 732: 233–247, 1994
58. Takanaga K, Nakamura Y, Endo H, Sakiyama S: Involvement of S100-related calcium-binding protein pEL98 (or mts1) in cell motility and tumor cell invasion. Jpn J Cancer Res 85: 831–839, 1994
59. Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, Sidransky D, Baylin SB: Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res 55: 4525–4530, 1995
60. Jarrard DF, Bova GS, Ewing CM, Pin SS, Nguyen SH, Baylin SB, Cairns P, Sidransky D, Herman JG, Isaacs WB: Deletional, mutational, and methylation analyses of CDKN2 (p16/MTS1) in primary and metastatic prostate cancer. Gen Chrom Canc 19: 90–96, 1997
61. Gao X, Porter AT, Honn KV: Involvement of the multiple tumor suppressor genes and 12-lipoxygenase in human prostate cancer. Therapeutic implications. Av Exp Med Biol 417: 41–53, 1997
62. Sun Y, Lin J, Katz AE, Fisher PB: Human prostatic carcinoma oncogene PTI-1 is expressed in human tumor cell lines and prostate carcinoma blood samples. Cancer Res 57: 18–23, 1997
63. Rinker-Schaeffer CW, Chekmareva MA, Mohler JL: The role of motility proteins and metastasis-suppressor genes in prostate cancer progression. Stem Cells 14: 508–516, 1996
64. Dong J-T, Isaacs WB, Isaacs JT: Molecular advances in prostate cancer. Curr Opin Oncology 9: 101–107, 1997
65. Fong CJ, Sutkowski DM, Kozlowski JM, Lee C: Utilization of the Boyden chamber to further characterize *in vitro* migration and invasion of benign and malignant human prostatic epithelial cells. Invas Metast 12: 264–274, 1992

66. Wang FL, Wang Y, Ong WK, Liu Y, Addivinola FJ, Liang P, Chen LB, Kantoff PW, Pardee AB: Two differentially expressed genes in normal human prostate tissue and in carcinoma. Cancer Res 56: 3634–3637, 1996
67. Smith P, Rhodes NP, Shortland AP, Fraser SP, Djamgoz MB, Ke Y, Foster CS: Sodium channel protein expression enhances the invasiveness of rat and human prostate cancer cells. FEBS Lett 423: 19–24, 1998
68. Viola MV, Fromowitz F, Oravez S, Deb S, Finkel G, Lundy J, Hand P, Thor A, Schlom J: Expression of *ras* oncogene p21 in prostate cancer. N Eng J Med 314: 133–137, 1986
69. Partin AW, Isaacs JT, Treiger B, Coffey DS: Early cell motility changes associated with an increase in metastatic ability in rat prostatic cancer cells transfected with the v-Harvey-*ras* oncogene. Cancer Res 48: 6050–6053, 1988
70. Cooke DB, Quarmby VE, Petrusz P, Mickey DD, Der CJ, Isaacs JT, French FS: Expression of *ras* proto-oncogenes in the Dunning R3327 rat prostatic adenocarcinoma system. Prostate 13: 273–287, 1988
71. Powell CT, Gschwend JE, Fair WR, Brittis NJ, Stec D, Huryk R: Overexpression of protein kinase C-zeta (PKC-zeta) inhibits invasive and metastatic abilities of Dunning R-3327 MAT-LyLu rat prostate cancer cells. Cancer Res 56: 4137–4141, 1996
72. Epner DE, Partin AW, Schalken JA, Isaacs JT, Coffey DS: Association of glyceraldehyde-3-phosphate dehydrogenase expression with cell motility and metastatic potential of rat prostatic adenocarcinoma. Cancer Res 53: 1995–1997, 1993

Address for offprints: Dr. Bruce R. Zetter, Department of Surgical Research, Enders 1074, Children's Hospital, 300 Longwood Ave, Boston, MA 02115, USA; *Tel:* 617 355 6376; *Fax:* 617 355 7043; *e-mail:* zetter@al.tch.harvard.edu

THYMOSIN β15 AS A PROGNOSTIC MARKER FOR HUMAN PROSTATE CANCER

L. Bao[1], M. Loda[2], B. Zetter[1]

[1]Dept. of Surgery and Cell Biology, Children's Hospital, Harvard Medical School, Boston, MA, USA

[2]Beth Israel Deaconess Medical Center, West Campus, Harvard Medical School, Boston, MA, USA

Prostate cancer is the commonest cancer in men and the second leading cause of cancer death in American men. The widespread use of prostate specific antigen (PSA) test has greatly improved the earlier detection of human prostate cancers. However, this early detection does not help much to predict which tumors will progress to the metastatic diseases. To search for molecules that could be used as prognostic markers for prostate cancer, we compared gene expression among Dunning rat prostatic carcinoma cell lines with varying metastatic potential and cloned a gene called thymosin beta 15 (TB15), a new member of the thymosin beta family, from a metastatic subclone. TB15 was not detected in most normal adult rat and human tissues, including prostate. *In situ* hybridization and immunohistochemical staining of human prostate specimens showed that both TB15 mRNA and protein levels were elevated in invasive and metastatic tumors and correlated positively with the Gleason grade, a common histological grading system of prostate cancer. Up to 5 years follow-up data from the patients we could obtain showed that all patients who died of metastatic prostate cancer had positive staining for TB15 at the time of diagnosis. In contrast, patients who were negative for TB15 are still alive with no evidence of disease. These data suggest that TB15 could serve as a molecular marker that is able to distinguish prostate cancers destined to progress to lethal metastatic disease from those with little likelihood of causing morbidity.

Thymosin β peptides as diagnostic tools in cancer. Bruce R. Zetter, Lloyd Hutchinson and Lere Bao. Children's Hospital, Harvard Medical School, Boston, MA USA.

The β thymosin family consists of at least 17 different members. In any species, several different β thymosins will be expressed, each independently regulated to appear in different cell types and at different times in development. Several β thymosins, including thymosin β4, β10, β15 and βNB have been shown to be upregulated in certain human cancers. We have made specific C-terminal antibodies that distinguish between thymosins β4, β10, β15, and βNB. Using prostate cancer as an example, we have shown that thymosins β15 and βNB are elevated in aggressive human prostate cancer whereas thymosin β4 levels are reduced. Additionally, we find that the upregulation of thymosin β15 in primary prostate cancer correlates with later recurrence of the disease and with the formation of distant metastasis. Thymosin β15 is more likely to be upregulated in invasive and metastatic cancers than in low-grade non-invasive cancers. Patients who have positive thymosin β15 staining at the time of diagnosis are 7 times more likely to have tumor recurrence than patients who are thymosin β15-negative. We are also able to detect thymosin β15 in the urine of patients with prostate cancer. Thymosin β15 levels drop after prostate removal surgery and rise again if there is local or distant recurrence of tumor growth. Our results therefore indicate that thymosin β15 is useful both as a marker for future outcome in patients with newly diagnosed cancer and as a tool for follow-up of prostate cancer patients to determine if tumor recurrence takes place.

Dual functions for Tβ15 in tumor progression: intracellular motility and extracellular angiogenic functions

L. M. Hutchinson, S. M. Short, C. M. Becker, S.Bakthavachalam, and B. Zetter.
Surgical Research Laboratory, Children's Hospital, Boston, MA 02115

Beta-thymosins are a family of proteins (e.g. Tβ15), which correlate with increasing tumor malignancy and metastatic potential. These proteins bind to G-actin, retard actin polymerization and influence cell motility. Tβ4 also acts as an extracellular factor, which promotes endothelial cell migration *in vitro*. We examined the β-thymosin profile in low and high metastatic variants derived from the same parent prostate carcinoma cell lines. Northern blot analysis of cells with increasing metastatic potential revealed changes in the β-thymosin composition characterized by rising levels of Tβ10 and Tβ15 with a simultaneous reduction in Tβ4. We asked if raising Tβ15 expression could enhance tumorgenesis, metastasis, or angiogenesis. Stable populations producing native Tβ15 (Tβ15wt) or a secreted form (secTβ15) were created from poorly metastatic variants of the Dunning carcinoma cell lines, which lack endogenous Tβ15. Western blot analysis revealed that both native Tβ15wt and secTβ15 proteins were released from cells into culture medium. Conditions permitting the release of G-actin from cells, coupled with size-dependent fractionation of conditioned media, showed that extracellular Tβ15 disassociated from the G-actin complex. This is consistent with the hypothesis that Tβ15 has intracellular and extracellular functions. *In vitro* growth rates of Tβ15wt, secTβ15 and vector control cells were nearly identical, whereas subcutaneous tumors producing Tβ15 in Dunning rats exhibited a higher growth rate. After one month, tumors expressing Tβ15 were 50% larger than the vector controls, indicating that addition of Tβ15 accelerates tumor expansion. Tβ15 synthesis triggered a dramatic increase in cell motility, boosting serum response by 3 to 10 fold, relative to vector control. However, changes in cell motility did not translate into elevated rates of tumor metastasis, suggesting other factors may be required to alter this process. Further, synthetic protein and natural forms of Tβ15 obtained from conditioned medium acted as endothelial chemoattractants *in vitro*, yet did not stimulate the migration of human fibroblasts or smooth muscle cells. Together these results confirm that Tβ15 is advantageous to tumor cells and points to a dual role in tumor progression: intracellular motility protein and extracellular angiogenic factor.

ELSEVIER

RAPID COMMUNICATION

THYMOSIN BETA-15 PREDICTS FOR DISTANT FAILURE IN PATIENTS WITH CLINICALLY LOCALIZED PROSTATE CANCER—RESULTS FROM A PILOT STUDY

ARNAB CHAKRAVARTI, ELIZABETH M. ZEHR, ANTHONY L. ZIETMAN, WILLIAM U. SHIPLEY, WILLIAM B. GOGGINS, DIANNE M. FINKELSTEIN, ROBERT H. YOUNG, ERIC L. CHANG, AND CHIN-LEE WU

ABSTRACT

Objectives. To report the results of a pilot study on the prognostic value of a newly identified actin-binding protein, thymosin beta-15 (Tβ15), in predicting prostate-specific antigen (PSA) and bone failure in patients with Gleason 6/10 clinically localized prostate cancer.

Methods. Thirty-two patients (median age 70 years) with clinically localized, moderately differentiated (Gleason 6/10) prostate cancer treated by external beam radiotherapy alone (68.4 Gy) with available paraffin blocks at the Massachusetts General Hospital were evaluated for this pilot study. All patients had clinical Stage M0 disease at initial presentation, which was documented by bone scan (T1c-4,NX). Their corresponding biopsy specimens were stained immunohistochemically for Tβ15, which was then correlated with the clinical outcome in a blinded manner. The median follow-up was 6 years (range 1 to 19) for all of the patients.

Results. The outcomes of the 32 patients can be grouped into three categories: patients with no evidence of disease (n = 11), patients with PSA failure without documented bone failure (n = 11), and patients with PSA failure and documented bone failure (n = 10). Tβ15 staining intensity strongly correlated with clinical outcome. Of those patients whose specimens stained 3+ (strongest staining), 62% developed bone failure compared with 13% of those patients whose specimens stained 1+ (weakest staining) ($P = 0.01$). The 5-year freedom from PSA failure was only 25% for those patients with 3+ staining compared with 83% for those with 1+ staining ($P = 0.02$).

Conclusions. The results of this pilot study have demonstrated that Tβ15 staining intensity may be a potentially important marker to identify high-risk patients with moderately differentiated, clinically localized prostate cancer. UROLOGY **55:** 635–638, 2000. © 2000, Elsevier Science Inc.

Despite aggressive radiotherapy, it is known that a substantial percentage of patients with clinical Stage M0 prostate cancer will have progression with distant metastases at 15 years.[1–5] Many of these patients probably had occult micrometastatic disease at presentation. From a therapeutic standpoint, it is important at the time of the initial diagnosis to identify patients who are at high risk of harboring micrometastatic disease because systemic therapy has potential value in eradicating micrometastatic disease.[6–8]

Gleason grade in its extremes does predict for distant metastases, but up to 80% of patients at their initial presentation have moderately differentiated tumors, which highlights the need for other prognostic markers in this group of patients. Cell motility is known to underlie many of the steps involved in the metastatic cascade. Bao *et al.*[9] have identified an actin-binding protein, thymosin beta-15 (Tβ15), as a potentially important marker for prostate cancer cells with high metastatic potential by enhancing cell motility.

A direct correlation between an increasing Gleason grade and positivity of staining has been re-

From the Departments of Radiation Oncology, Biostatistics, and Pathology and Urology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts; and Department of Radiation Oncology, M.D. Anderson Cancer Center, Houston, Texas

Reprint requests: Arnab Chakravarti, M.D., Department of Radiation Oncology, Massachusetts General Hospital, Harvard Medical School, 100 Blossom Street, Cox Building-Basement, Boston, MA 02114

Submitted: December 14, 1999, accepted (with revisions): December 29, 1999

© 2000, ELSEVIER SCIENCE INC.
ALL RIGHTS RESERVED

0090-4295/00/$20.00
PII S0090-4295(00)00462-3

ported.[9] Interestingly, for patients with moderately differentiated tumors (Gleason 6-7), a wide range of staining intensities was observed, with specimens staining strongly, weakly, or not at all. This raises the possibility that Tβ15 staining can identify which patients with moderately differentiated tumors are at greatest risk of harboring micrometastatic disease and, hence, at greatest risk of subsequent development of biochemical and/or clinically evident bone failure. As the vast majority of patients at initial presentation have moderately differentiated tumors, this is a clinically important question.

This is the first reported clinical study to our knowledge to determine, in a blinded manner, whether Tβ15 staining of the initial biopsy specimen predicts for the subsequent development of biochemical and/or bone failure for patients with moderately differentiated prostate cancers who had clinical Stage M0 disease at the initial presentation. This study was designed to be a pilot study consisting of a relatively modest number of patients, which would lead to a larger scale multi-institutional study if a positive correlation were found.

MATERIAL AND METHODS

Three hundred patients with moderately differentiated prostate adenocarcinoma (Gleason grade 3 of 5 and score 6 of 10) treated by external beam radiation and with up-to-date follow-up data were identified from the Massachusetts General Hospital Radiation Oncology prostate cancer data base. Thirty-two of these patients had available paraffin blocks from the initial biopsy at Massachusetts General Hospital for analysis. The clinical staging was determined using the AJCC criteria, and all patients had Stage M0 disease at the initial presentation, as confirmed by bone scan. The distribution of patients by clinical T stage was as follows: T1c, 3; T2a, 2; T2b, 11; T3, 13; T4, 2; and TX, 1. The median pretreatment prostate-specific antigen (PSA) level was 7.1 ng/mL (range 1.0 to 49.9). The median age for all patients was 70 years (range 63 to 86). The median follow-up was 6 years (range 1 to 19). The patients were treated with external beam irradiation alone to a total dose of 68.4 Gy using high-energy photon irradiation. No systemic therapy, hormonal or chemotherapy, was rendered to these patients.

The corresponding tissue specimens from the initial biopsy (before treatment) were obtained for all 32 patients. All specimens were fixed in formalin and embedded in paraffin. On review by light microscope, all residual tissue contained moderately differentiated adenocarcinoma, Gleason score 6/10. Immunohistochemical staining was performed as previously described by the use of avidin-biotin-conjugated peroxidase complex technique (Vector Laboratories, Burlingame, Calif). Tβ15 antibody was a generous gift from L. Bao and B. Zetter (Children's Hospital, Boston, Mass). Sections without primary antibody or sections with benign prostatic tissue were used as negative controls. Staining and interpretation of staining were done without knowledge of the clinical outcome.

RESULTS

The outcomes of these 32 patients can be grouped into three categories: patients with no evidence of disease (n = 11), patients with PSA failure without documented bone failure (n = 11), and patients with PSA failure and documented bone failure (n = 10). PSA failure was defined as three consecutive post-treatment rises. Bone failure, when present, was documented by bone scan. Of the 11 patients with PSA failure without bony metastases, 5 had received hormonal therapy at the time of the initial PSA failure. Of the 10 patients with PSA failure and documented bone failure, only 1 had received hormonal therapy when the PSA elevation was first detected. Therefore, hormonal therapy likely delayed the appearance of distant metastases in these 5 patients with PSA elevation without evident bony metastases.

Normal prostatic acinar epithelium and benign hyperplastic epithelium stained negatively for Tβ15 (Fig. 1A), confirming the observation made by Bao *et al.*[9] Interestingly, endothelial cells within the biopsy specimens stained with moderate intensity for Tβ15, which was comparable in all specimens. However, the invariability of the presence of blood vessels in the tissue and the consistent endothelial staining with Tβ15 antibody prompted us to use endothelial staining as a reliable positive internal control and a reference point for scoring tumor cell staining. When most of the tumor cells (greater than 70%) stained with lower intensity than the endothelial cells, the specimen was graded negative/weakly positive (1+) (Fig. 1B). When most of the tumor cells (greater than 70%) stained with equivalent intensity to the endothelial cells or had a mixed staining pattern, the specimen was scored as moderate staining (2+) (Fig. 1C). When most of the tumor cells (greater than 70%) stained more intensely than the endothelial cells, the specimen was graded as strongly positive (3+) (Fig. 1D).

A Tβ15 stain of 3+ strongly predicted the subsequent development of PSA failure with bony metastases. For all 32 patients, 8 (62%) of the 13 patients with 3+ staining developed bony metastasis compared with only 1 (13%) of 8 patients with 1+ staining and 1 (14%) of 7 patients with 2+ staining ($P = 0.01$ by exact trend test).

At 5 years of follow-up, a Tβ15 staining score of 3+ strongly predicted for PSA failure. For all 25 patients followed up for more than 5 years, the 5-year actuarial biochemical control rate was 44%. For a Tβ15 stain of 1+, 2+, or 3+, the corresponding 5-year actuarial biochemical control rate was 83% (n = 6), 43% (n = 7), and 25% (n = 12), respectively ($P = 0.02$ by exact trend test). At 5 years, the positive predictive value for a Tβ15 stain score of 3+ was 86% (with biochemical failure as the end point). The negative predictive value for a Tβ15 stain score of 1+ was 71% (with freedom from biochemical failure as the end point).

To control for confounding by other predictors of

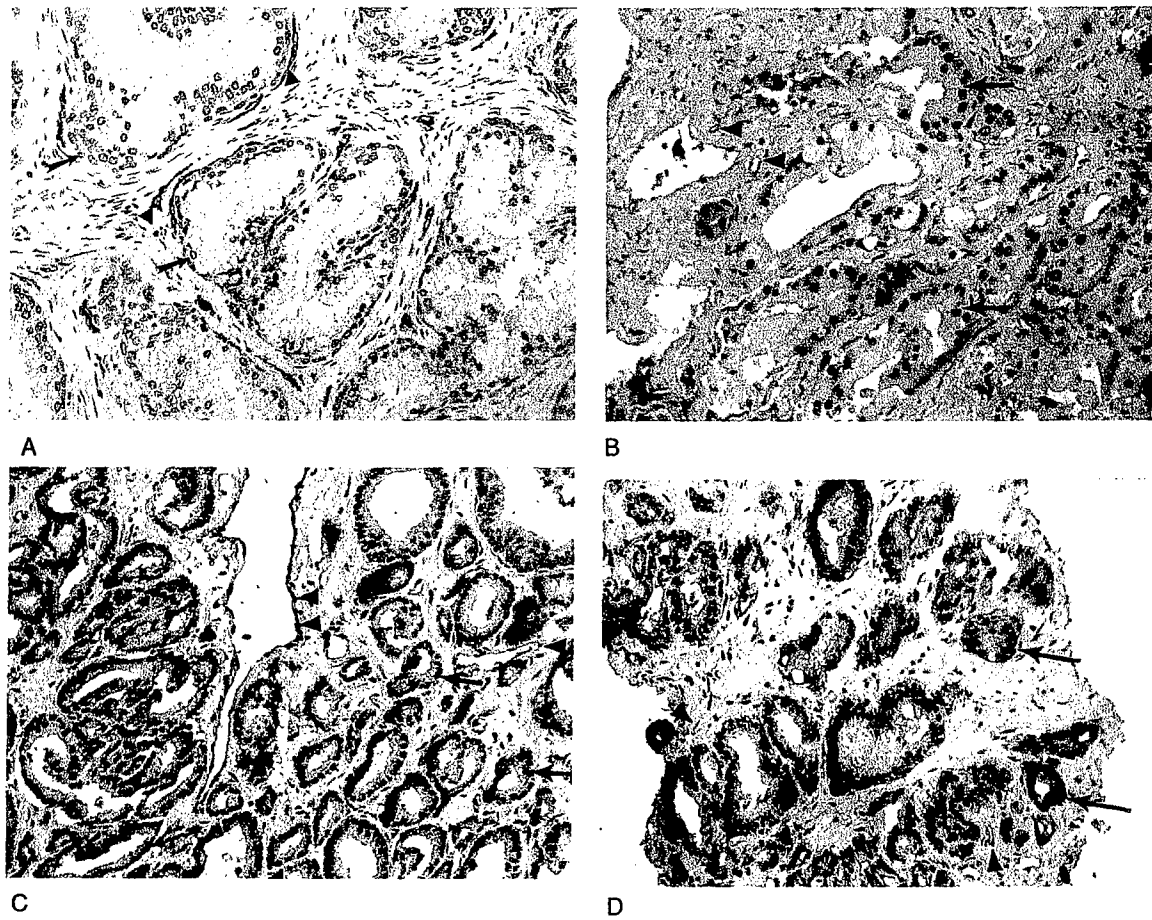


FIGURE 1. *Immunohistochemical staining of Tβ15 on prostatic tissue. Affinity purified anti-Tβ15 antibody was used to stain (A) benign prostatic tissue and (B to D) Gleason grade 3/5 prostate adenocarcinoma. (A) Benign glandular cells (arrows) did not stain. In contrast, vascular endothelial cells (arrowheads) between the acini showed positive staining. (B) Acinar tumor cells (arrows) showed only weak focal staining and the endothelial cells (arrowheads) stained positively, indicating a true negative result. (C) Tumor cells (arrows) and endothelial cells (arrowheads) showed roughly equal staining intensity. (D) Tumor cells (arrows) show diffuse stronger staining compared with the endothelial cells (arrowheads).*

failure, a multiple logistic regression model was fit to the data. Other covariates that were simultaneously put in the model were T stage, pretreatment PSA, age, and the subsequent development of local failure. The subsequent development of local failure ($P = 0.34$) and PSA ($P = 0.59$) were not significant predictors and were dropped from the final model. The final model included age ($P = 0.18$), T stage ($P = 0.08$), and staining intensity ($P = 0.05$). This analysis reveals that Tβ15 staining predicted for PSA failure independent of the other factors. The adjusted odds ratio for staining was 4.35, with a 95% confidence interval of 1.01 to 18.86. Thus, for each unit increase in staining value (from 1 to 2 or 2 to 3), the odds of developing biochemical failure increase by an estimated factor of 4.35.

COMMENT

Metastases can occur quite early in the course of human prostate cancer. A recent study revealed that more than one half of patients with pathologically organ-confined disease had positive bone marrow specimens by reverse transcriptase-polymerase chain reaction analysis.[10] These data, along with clinical observations, support the hypothesis that early dissemination of prostate cancer may occur despite local control of the disease.

Given the magnitude of this problem, molecular markers have been sought to identify high-risk patients. Low expression of cell cycle regulatory proteins such as p53, pRB, and p27 and high expression of Ki-67 have been found to predict an unfavorable outcome in patients with prostate cancer.[11–13] It has been demonstrated that time-lapse videomicroscopic images can distinguish high from low metastatic potential cell lines when membrane ruffling, pseudopodal extension, and cellular translations of single cells are analyzed.[14] Hence, enhanced cell motility, perhaps Tβ15-dependent, underlies the metastatic process.

Tβ15 mRNA is absent in most normal tissues, with the exception of the testes.[15] Other tumor cell lines, including highly metastatic prostate, lung, breast, and melanoma cell lines, strongly express Tβ15. Corresponding cell lines with low metastatic potential have low levels or absent Tβ15 staining.

The wide range of Tβ15 staining intensities among cases of moderately differentiated tumors raised the question of whether Tβ15 staining can effectively discriminate between moderately differentiated tumors that are at high risk of harboring occult metastases from those at lower risk. The results from the 32 patients in this study with Gleason score 6/10 tumors reveal that at 5 years of follow-up, a Tβ15 stain of 3+ has a high positive predictive value (86%) for the subsequent development of PSA failure. Likewise, at 5 years, a Tβ15 stain of 1+ has a high negative predictive value (71%), with freedom from PSA failure as the end point.

The results of this pilot study suggest that Tβ15 staining of the initial biopsy specimens from patients with Stage M0 moderately differentiated prostate adenocarcinoma has strong predictive value for the subsequent development of biochemical and clinically evident bone failure. Further investigations on a larger patient population are planned.

REFERENCES

1. Bagshaw MA, Kaplan ID, and Cox RC: Radiation therapy for localized disease. Cancer 71: 939–952, 1993.

2. Hanks GE, Krall JM, and Hanton AL: Patterns of care and RTOG studies in prostate cancer: long-term survival, hazard rate observations and possibilities of cure. Int J Radiat Oncol Biol Phys 28: 39–45, 1993.

3. Perez CA, Lee HK, Georgiou A, *et al:* Technical and tumor-related factors affecting outcome of definitive irradiation for localized carcinoma of the prostate. Int J Radiat Oncol Biol Phys 26: 581–591, 1993.

4. Zagars GK, von Escenbach AC, and Ayala AG: Prognostic factors in prostate cancer: analysis of 874 patients treated with radiation therapy. Cancer 72: 1709–1725, 1993.

5. Zietman AL, Prince EA, Nakfoor BM, *et al:* Neoadjuvant androgen suppression with radiation in the management of locally advanced adenocarcinoma of the prostate: experimental and clinical results. Urology 49(suppl 3A): 74–83, 1997.

6. Pilepich MV, Krall JM, Al-Sarraf M, *et al:* Androgen deprivation with radiation therapy alone for locally advanced prostatic carcinoma: a randomized comparative trial of the Radiation Therapy Oncology Group. Urology 45: 616–623, 1995.

7. Duchesne GM, Bloomfield D, and Wall P: Identification of intermediate-risk prostate cancer patients treated with radical radiotherapy suitable for neoadjuvant hormone studies. Radiother Oncol 38: 7–12, 1996.

8. Bolla M, Gonzalez D, Warde P, *et al:* Improved survival in patients with locally advanced prostate cancer treated with radiotherapy and goserelin. N Engl J Med 337: 295–300, 1997.

9. Bao L, Loda M, Janmey P, *et al:* Thymosin β15: a novel regulator of tumor cell motility upregulated in metastatic prostate cancer. Nat Med 12: 1322–1328, 1996.

10. Melchior S, Corey E, Ellis W, *et al:* Early tumor cell dissemination in patients with clinically localized carcinoma of the prostate. Clin Cancer Res 3: 249–256, 1997.

11. Theodorescu D, Broder SR, Boyd JC, *et al:* p53, bcl-2, and retinoblastoma proteins as long-term prognostic markers in localized carcinoma of the prostate. J Urol 158: 131–137, 1997.

12. Tsihlias J, Kapusta LR, DeBoer G, *et al:* Loss of cyclin-dependent kinase inhibitor $p27^{kip1}$ is a novel prognostic factor in localized human prostate adenocarcinoma. Cancer Res 58: 542–548, 1998.

13. Mashal RD, Lester S, Corless C, *et al:* Expression of cell cycle-regulated proteins in prostate cancer. Cancer Res 56: 4159–4163, 1996.

14. Mohler J, Partin A, Sharief Y, *et al:* Prostate cancer cell motility and metastasis. J Cell Biochem Suppl 19B: 8, 1995.

15. Bao L, Loda M, and Zetter B: Thymosin β15 expression in tumor cell lines with varying metastatic potential. Clin Exp Metastasis 16: 227–233, 1998.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 17 Jun 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for grants. Request the limited distribution statements for the Accession Documents listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

ACCESSION DOCUMENT NUMBERS

ADB266028

ADB259887

ADB259894

ADB259837

ADB258947

ADB274390

ADB262488

ADB257222

ADB274382

ADB258931

~~ADB260157~~

~~ADB274[illegible]~~ > Do not downgrade